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Award Number: DAMD17-01-1-0394

TITLE: AKT2 Oncogene and Phosphoinositide 3-Kinase in Human Breast Cancer

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REPORT DATE: September 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2004	Final (20 Aug 2001 - 19 Aug 2004)

4. TITLE AND SUBTITLE
AKT2 Oncogene and Phosphoinositide 3-Kinase in Human Breast Cancer

5. FUNDING NUMBERS
DAMD17-01-1-0394

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

By screening NCI diversity set that was derived from 140,000 compounds, we have identified several potential Akt inhibitors, one of which has been further characterized and named Akt/PKB inhibitor (API)-2. API-2 specifically inhibited kinase activity of AKT1, AKT2 and AKT3. The inhibition of Akt kinase resulted in suppression of cell growth and induction of apoptosis in human cancer cells that harbor constitutively activated Akt due to overexpression of Akt or other genetic alterations such as PTEN mutation. Furthermore, API-2 potently inhibited tumor growth in nude mice of human cancer cells where Akt is aberrantly expressed/activated but not of those cancer cells where it is not. In addition, we have demonstrated Akt phosphorylation of XIAP to exert its cell survival signal at postmitochondrial level. We have also shown that Akt is targeted by geranylgeranyltransferase I inhibitors (GGTIs).

14. SUBJECT TERMS
AKT2, PI3K, inhibitor, XIAP, GGTI, apoptosis

15. NUMBER OF PAGES
43

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

The purpose of this project is to: 1) determine the incidence and clinicopathological significance of *PI3K/AKT2* alterations in breast cancer, 2) examine the role of overexpression of active and wild type PI3K/AKT2 in mammary cell transformation and 3) determine the role of PI3K/AKT2 in chemoresistance and as targets for breast cancer intervention.

Body:

During the last budget year, we have mainly focused our effort on determine the role of PI3K/AKT2 in as targets for breast cancer intervention and mechanism of AKT2 in mammary epithelial transformation.

1. Develop a potent Akt/PKB inhibitor (API)-2 with anti-tumor activity *in vitro* and *in vivo.*

We have previously shown frequent activation and/or overexpression of PI3K and AKT2 in human breast cancer. Activation of the Akt pathway plays a pivotal role in malignant transformation and chemoresistance by inducing cell survival, growth, migration and angiogenesis. Therefore, Akt is believed to be a critical target for cancer intervention including breast carcinoma. To identify Akt inhibitor(s), we have screened NCI diversity set that was derived from the 140,000 compounds. Thirty-two compounds were obtained and shown to significantly inhibit growth in AKT2-transformed but not in pcDNA3-transfected NIH 3T3 cells. Further analyses show that two of them directly inhibited constitutively active AKT2 kinase activity and another two directly decrease AKT phosphorylation in cultured cells. All four compounds significantly inhibit growth in 3 human cancer cell lines where AKT pathway is altered. One compound, named API (Akt/PKB signaling inhibitor)-2, has been further characterized. API-2 suppressed the kinase activity and phosphorylation level of Akt. The inhibition of Akt kinase resulted in suppression of cell growth and induction of apoptosis in human cancer cells that harbor constitutively activated Akt due to overexpression of Akt or other genetic alterations such as PTEN mutation. API-2 is highly selective for Akt and does not inhibit the activation of PI3K, PDK1, PKC, SGK , PKA, STAT3, Erk-1/2, or JNK. Furthermore, API-2 potently inhibited tumor growth in nude mice of human cancer cells where Akt is aberrantly expressed/activated but not of those cancer cells where it is not. These findings provide strong evidence for pharmacologically targeting Akt for anticancer drug discovery. API-2 could be a potential chemotherapeutic drug for breast cancer treatment (3, See Appendix).

2. Akt phosphorylation and stabilization of X-linked inhibitor of apoptosis, XIAP: a mechanism of Akt anti-apoptosis at postmitochondria level

Akt negatively regulates apoptotic pathways at a pre-mitochondrial level through phosphorylation and modulation of proteins such as Bad, NFκB, Forkhead proteins and GSK-3β (4–9). Akt has also been shown to protect cell death at a post-mitochondrial level (10), although its downstream targets have not been well documented. Here, we demonstrate that Akt, including AKT1 and AKT2, interacts with and phosphorylates XIAP at residue serine-87 *in vitro* and *in vivo*. Phosphorylation of XIAP by Akt protects XIAP from ubiquitination and degradation in response to cisplatin. Moreover, autoubiquitination of XIAP is also inhibited by Akt. Consistent with this, an XIAP mutant introduced into cells which mimics the Akt-phosphorylated form (i.e., XIAP-S87D) displays reduced ubiquitination and degradation as compared to wild type XIAP. The greater stability of XIAP-S87D in cells translated to increased cell survival after cisplatin treatment.

Conversely, a mutant that could not be phosphorylated by Akt (XIAP-S87A) was more rapidly degraded and showed increased cisplatin-induced apoptosis. In addition, knockdown XIAP by RNA interference and adenovirus-antisense of XIAP largely abrogated Akt-inhibited cell death in response to cisplatin treatment. These data identify XIAP as a new downstream target of Akt and an important mediator of Akt's effect on cell survival (11, See Appendix).

3. Geranylgeranyltransferase I Inhibitor-298 inhibits AKT2 and survivin pathway to overcome chemoresistance.

GGTI represents a new class of anti-cancer drugs that show promise in blocking the tumor growth (12). However, the mechanism by which GGTIs contribute to inhibit tumor cell proliferation is still unclear. We have recently demonstrated that GGTI-298 induces apoptosis in both cisplatin sensitive and resistant human cancer cells by inhibition of PI3K/Akt, including AKT1 and AKT2, and survivin pathways. Following GGTI-298 or GGTI-2166 treatment, kinase levels of PI3K and AKT were decreased and survivin expression was significantly reduced. Ectopic expression of constitutively active AKT2 and/or survivin significantly rescue human cancer cells from GGTI-298-induced apoptosis. Previous studies have shown that Akt mediates growth factor-induced survivin, whereas p53 inhibits survivin expression. However, constitutively active AKT2 failed to rescue the GGTIs downregulation of survivin. Further, GGTIs suppress survivin expression and induce programmed cell death in both wild type p53 and p53-deficient ovarian cancer cell lines. These data indicate that GGTI-298 and GGTI-2166 induce apoptosis by targeting PI3K/AKT and survivin parallel pathways independent of p53. Due to the fact that upregulation of Akt and survivin as well as inactivation of p53 are frequently associated with chemoresistance (13-15), GGTI could be valuable agents to overcome anti-tumor drug resistance (16, See Appendix).

Key Research Accomplishment

1 Identification of a specific Akt inhibitor, API-2, with anti-tumor activity.
2 Akt, including AKT1 and AKT2 interaction with and phosphorylation of XIAP to exert its cell survival function at post-mitochondrial level.
3 Akt is targeted by geranylgeranyltransferase I Inhibitor.

Reportable Outcomes

Yang L., Dan H.C., Sun M., Liu Q., Sun X., Feldman R.I., Hamilton A.D., Polokoff M., Nicosia S.V., Herlyn M., Sebti S.M., and Cheng, J.Q. Akt/Protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt.. *Cancer Res.* 64:4394-4399, 2004

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Conclusion

1. API-2 is a specific Akt inhibitor and has a great potential for treatment of breast tumors exhibiting elevated level of Akt.
2. XIAP is a physiological substrate of Akt and mediates Akt survival signal at postmitochondrial level.
3. GGTI is a potential drug for overcoming cisplatin resistance in human cancer.

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Appendices

1. Yang L., Dan H.C., Sun M., Liu Q., Sun X., Feldman R.I., Hamilton A.D., Polokoff M., Nicosia S.V., Herlyn M., Sebti S.M., and Cheng, J.Q. Akt/Protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt.. *Cancer Res.* 64:4394-4399, 2004

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[CANCER RESEARCH 64, 4394–4399, July 1, 2004]

Advances in Brief

Appendix 1

Akt/Protein Kinase B Signaling Inhibitor-2, a Selective Small Molecule Inhibitor of Akt Signaling with Antitumor Activity in Cancer Cells Overexpressing Akt

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Abstract

Accumulated studies have shown that activation of the Akt pathway plays a pivotal role in malignant transformation and chemoresistance by inducing cell survival, growth, migration, and angiogenesis. Therefore, Akt is believed to be a critical target for cancer intervention. Here, we report the discovery of a small molecule Akt pathway inhibitor, Akt/protein kinase B signaling inhibitor-2 (API-2), by screening the National Cancer Institute Diversity Set. API-2 suppressed the kinase activity and phosphorylation level of Akt. The inhibition of Akt kinase resulted in suppression of cell growth and induction of apoptosis in human cancer cells that harbor constitutively activated Akt due to overexpression of Akt or other genetic alterations such as PTEN mutation. API-2 is highly selective for Akt and does not inhibit the activation of phosphatidylinositol 3′-kinase, phosphoinositide-dependent kinase-1, protein kinase C, serum- and glucocorticoid-inducible kinase, protein kinase A, signal transducer and activators of transcription 3, extracellular signal-regulated kinase-1/2, or c-Jun NH_2-terminal kinase. Furthermore, API-2 potently inhibited tumor growth in nude mice of human cancer cells in which Akt is aberrantly expressed/activated but not of those cancer cells in which it is not. These findings provide strong evidence for pharmacologically targeting Akt for anticancer drug discovery.

Introduction

Akt, also named protein kinase B, represents a subfamily of the serine/threonine kinase. Three members, AKT1, AKT2, and AKT3, have been identified in this subfamily. Akt is activated by extracellular stimuli in a phosphatidylinositol 3′-kinase (PI3k)-dependent manner (1, 2). Full activation of Akt requires phosphorylation of threonine 308 in the activation loop and serine 473 in the COOH-terminal activation domain. Akt is negatively regulated by PTEN tumor suppressor. Mutations in *PTEN* have been identified in various tumors, which lead to activation of Akt pathway (1, 2). In addition, amplification, overexpression, and/or activation of Akt have been detected in a number of human malignancies (1, 2). Ectopic expression of Akt, especially constitutively active Akt, induces cell survival and malignant transformation, whereas inhibition of Akt activity stimulates apoptosis in a range of mammalian cells (1–4). Furthermore, activation of Akt has been shown to associate with tumor invasiveness and chemoresistance (5). These observations establish Akt as an attractive target for cancer therapy.

Here, we report the identification of a small molecule inhibitor of Akt pathway, Akt/protein kinase B signaling inhibitor-2 (API-2), by screening the National Cancer Institute (NCI) Diversity Set. API-2 potently inhibits Akt signaling in human tumor cells with aberrant Akt, leading to inhibition of cell growth and induction of apoptosis. In a xenograft nude mice model, API-2 significantly inhibits tumor growth in Akt-overexpressing cells but not in the tumors with low levels of Akt.

Materials and Methods

Cell Lines and NCI Diversity Set. All cell lines used in this study were either purchased from American Type Culture Collection or described previously (4, 6). The NCI Structural Diversity Set is a library of 1,992 compounds selected from the approximately 140,000-compound NCI drug depository. In-depth data on the selection, structures, and activities of these diversity set compounds can be found on the NCI Developmental Therapeutics Program Web site.[5]

Screening for Inhibition of Akt-Transformed Cell Growth. AKT2 transformed NIH3T3 cells or LXSN vector-transfected NIH3T3 control cells (4) were plated into 96-well tissue culture plate. After treatment with 5 μM NCI Diversity Set compound, cell growth was detected with CellTier 96 One Solution Cell Proliferation kit (Promega). Compounds that inhibit growth in AKT2-transformed but not LXSN-transfected NIH3T3 cells were considered as candidates of Akt inhibitor and subjected to additional analysis.

***In Vitro* Protein Kinase, Cell Survival, and Apoptosis Assays.** *In vitro* kinase was performed as described previously (7). Cell survival was assayed with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (Promega). Apoptosis was detected with annexin V, which was performed as described previously (7). Recombinant Akt and phosphoinositide-dependent kinase-1 (PDK1) were purchased from Upstate Biotechnology, Inc.

Antitumor Activity in the Nude Mouse Tumor Xenograft Model. Tumor cells were harvested, resuspended in PBS, and injected s.c. into the right and left flanks (2×10^6 cells/flank) of 8-week-old female nude mice as reported previously (8). When tumors reached about 100–150 mm^3, animals were randomized and dosed i.p. with 0.2-ml vehicle of drug daily. Control animals received DMSO (20%) vehicle, whereas treated animals received injections of API-2 (1 mg/kg/day) in 20% DMSO.

Results

Identification of Small Molecule Inhibitor of Akt Signaling Pathway, API-2. Frequent alterations of Akt have been detected in human cancer, and disruption of Akt pathway induces apoptosis and inhibits tumor growth (9). Thus, Akt has been considered as an attractive molecular target for development of novel cancer therapeutics. To identify small molecule inhibitor(s) of Akt, we have evaluated a chemical library of 1,992-compounds from the NCI Diversity Set for agents capable of inhibition of growth in AKT2-transformed but not empty vector LXSN-transfected NIH3T3 cells as described in "Materials and Methods." Repeated experiments showed that 32 compounds inhibited growth only in AKT2-transformed cells. The most potent of these compounds, API-2 (NCI identifier, NSC 154020), suppressed cell growth at a concentration of 50 nM. Fig. 1*A* shows the chemical structure of API-2, which is also known as triciribine (TCN;

Received 2/2/04; revised 4/8/04; accepted 5/11/04.

Grant support: National Cancer Institute and Department of Defense (J. Q. Cheng).

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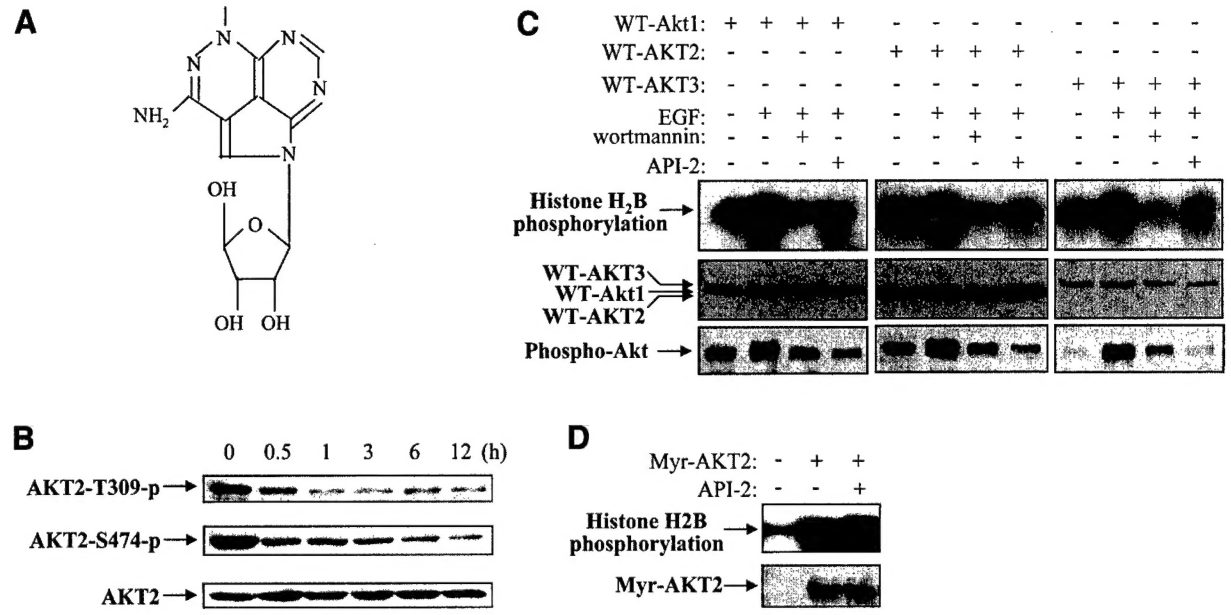

Fig. 1. Identification of API-2 (TCN) as a candidate of Akt inhibitor from the NCI Diversity Set. *A*, chemical structure of API-2 (TCN). *B*, API-2 inhibits phosphorylation levels of AKT2 in AKT2-transformed NIH3T3 cells. Wild-type (*WT*) AKT2-transformed NIH3T3 cells were treated with API-2 (1 μM) for indicated times and subjected to immunoblotting analysis with anti-phospho-Akt-threonine 308 (*AKT2-T309-p*) and anti-phospho-Akt-serine 473 (*AKT2-S474-p*) antibodies (*top* and *middle panels*). *Bottom panel* shows expression of total AKT2. *C*, API-2 inhibits three isoforms of Akt. HEK293 cells were transfected with HA-Akt1, -AKT2, and -AKT3 and treated with API-2 (1 μM) or wortmannin (15 μM) before EGF stimulation; the cells were lysed and immunoprecipitated with anti-HA antibody. The immunoprecipitates were subjected to *in vitro* kinase assay (*top*) and immunoblotting analysis with anti-phospho-Akt-threonine 308 (*bottom*) antibody. *Middle panel* shows expression of transfected Akt1, AKT2, and AKT3. *D*, API-2 did not inhibit Akt *in vitro*. *In vitro* kinase assay of constitutively active AKT2 recombinant protein in a kinase buffer containing 1 μM API-2 (*Lane 3*).

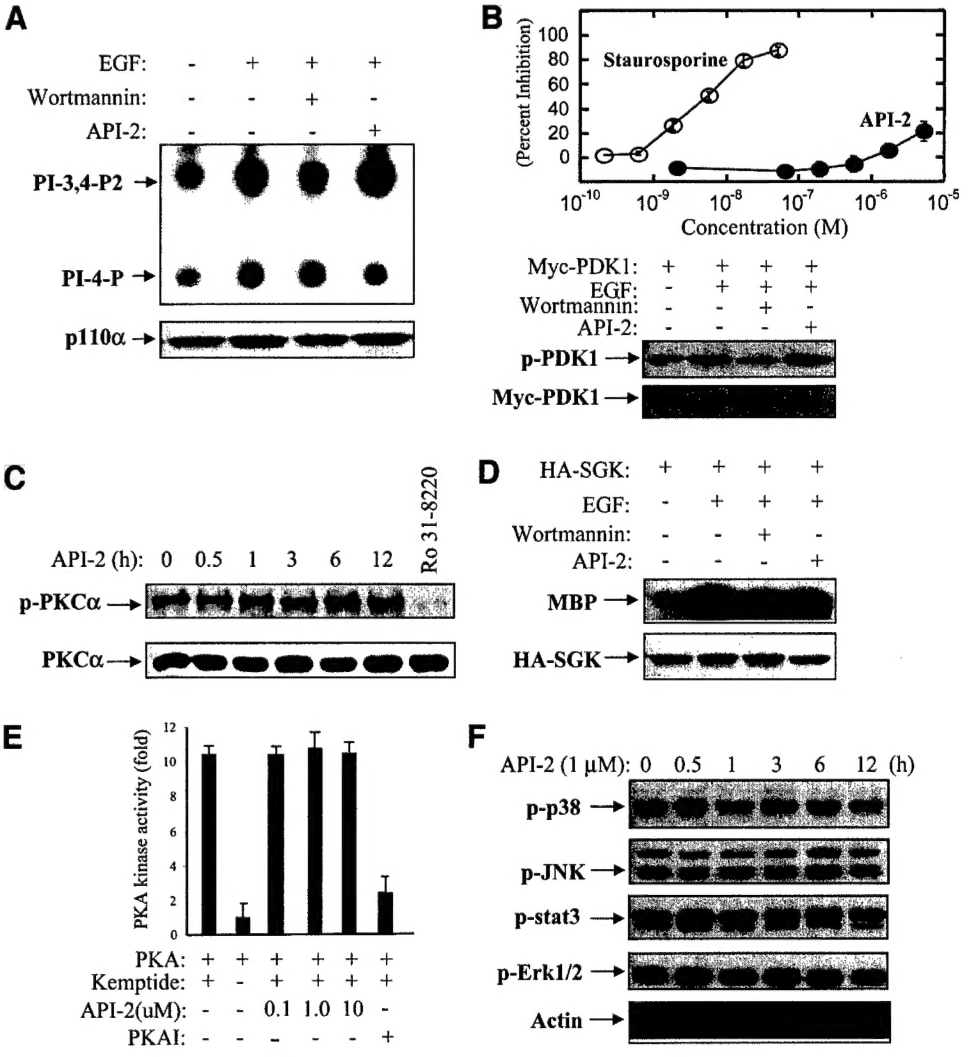

Fig. 2. API-2 does not inhibit PI3k, PDK1, and the closely related members of AGC kinase family. *A, in vitro* PI3k kinase assay. HEK293 cells were serum-starved and treated with API-2 (1 μM) or wortmannin (15 μM) for 30 min before EGF stimulation. Cells were lysed and immunoprecipitated with anti-p110α antibody. The immunoprecipitates were subjected to *in vitro* kinase assay using phosphatidylinositol 4-phosphate as substrate. *B*, effect of API-2 on *in vitro* PDK1 activation (*top panel*). ●, inhibition by API-2. ○, inhibition by the positive control staurosporine, which is a potent PDK1 inhibitor (IC_{50}, 5 nM). *Bottom panels* are immunoblotting analyses of HEK293 cells that were transfected with Myc-PDK1 and treated with wortmannin or API-2 before EGF stimulation. The immunoblots were detected with indicated antibodies. *C*, immunoblotting analysis of phosphorylation levels of PKCα with anti-phospho-PKCα-T638 (*top*) and total PKCα (*bottom*) antibodies after treatment with API-2 or a nonselective PKC inhibitor Ro31–8220. *D, in vitro* SGK kinase assay. HEK293 cells were transfected with HA-SGK and treated with API-2 or wortmannin before EGF stimulation. *In vitro* kinase was performed with HA-SGK immunoprecipitates using MBP as substrate (*top*). *Bottom panel* shows the expression of transfected HA-SGK. *E*, PKA kinase assay. Immuno-purified PKA was incubated in ADB buffer (Upstate Biotechnology, Inc.) containing indicated inhibitors (API-2 or PKAI) and substrate Kemptide. The kinase activity was quantified. *F*, Western blot. OVCAR3 cells were treated with API-2 for indicated times. Cell lysates were immunoblotted with indicated anti-phospho-antibodies (*panels 1–4*) and anti-actin antibody (*bottom*).

Ref. 10). The fact that API-2 inhibited selectively AKT-2-transformed cells over untransformed parental cells prompted us to determine whether API-2 is an inhibitor of AKT2 kinase. To this end, AKT2 was immunoprecipitated with anti-AKT2 antibody from AKT-2-transformed NIH3T3 cells after treatment with API-2. AKT2 immunoprecipitates were immunoblotted with anti-phospho-Akt antibodies. As shown in Fig. 1*B*, API-2 significantly inhibited AKT2 phosphorylation at both threonine 309 and serine 474, which are required for full activation of AKT2 (1, 2). Because three isoforms of Akt share high homology and similar structure, we next evaluated the effect of API-2 on their kinase activities. HEK293 cells were transfected with hemagglutinin (HA)-Akt1, HA-AKT2, and HA-AKT3; serum-starved overnight; and treated with API-2 for 60 min before epidermal growth factor (EGF; 50 ng/ml) stimulation. Triple experiments showed that API-2 suppressed EGF-induced kinase activity and phosphorylation of Akt1, AKT2, and AKT3 (Fig. 1*C*). However, kinase activity of recombinant constitutively active AKT2 (Myr-AKT2) was not inhibited by API-2 in an *in vitro* kinase reaction (Fig. 1*D*), suggesting that API-2 does not directly inhibit Akt *in vitro* and that API-2 neither functions as ATP competitor nor as the substrate competitor that binds to active site of Akt.

API-2 Does Not Inhibit Known Upstream Activators of Akt. It has been well documented that Akt is activated by extracellular stimuli and intracellular signal molecules, such as active Ras and Src, through a PI3k-dependent manner. Therefore, API-2 inhibition of Akt could result from targeting upstream molecule(s) of Akt. Because PI3k and PDK1 are direct upstream regulators of Akt (1, 2), we next examined whether API-2 inhibits PI3k and/or PDK1. HEK293 cells were serum-starved and then treated with API-2 or PI3k inhibitor wortmannin for 30 min before EGF stimulation. PI3k was immunoprecipitated with anti-p110α antibody. The immunoprecipitates were subjected to *in vitro* PI3k kinase assay using phosphatidylinositol 4-phosphate as a substrate. As shown in Fig. 2*A*, the EGF-induced PI3k activity was inhibited by wortmannin but not by API-2. To evaluate the effect of API-2 on PDK1, we used an assay in which recombinant PDK1 promotes the threonine 309 phosphorylation of AKT2 peptides in the presence of lipid vesicles containing phosphatidylinositol (2). As shown in Fig. 2*B*, the assay was potently inhibited by the control PDK1 inhibitor staurosporine (IC_{50}, 5 nM). In contrast, API-2 displayed only 21% inhibition of the assay at the highest concentration tested (5.1 μM). These data demonstrate that API-2 is not a potent inhibitor of PDK1. To further evaluate the effect of API-2 on PDK1 activation, we examined autophosphorylation level of PDK1 at serine 241, a residue that is phosphorylated by itself and is critical for its activity (1), after API-2 treatment of HEK293 cells. Triplicate experiments show that phosphorylation levels of PDK1 were not inhibited by API-2 (Fig. 2*B*). However, PI3k inhibitor wortmannin, as expected, inhibited EGF-stimulated PDK1 (Fig. 2*B*).

API-2 Is Highly Selective for the Akt over Protein Kinase C (PKC), Protein Kinase A (PKA), Serum- and Glucocorticoid-Inducible Kinase (SGK), Signal Transducer and Activators of Transcription, c-Jun NH_2-Terminal Kinase, p38, and Extracellular Signal-Regulated Kinase Signaling Pathways. Akt belongs to the AGC (PKA/PKG/PKC) kinase family, which also includes PKA,

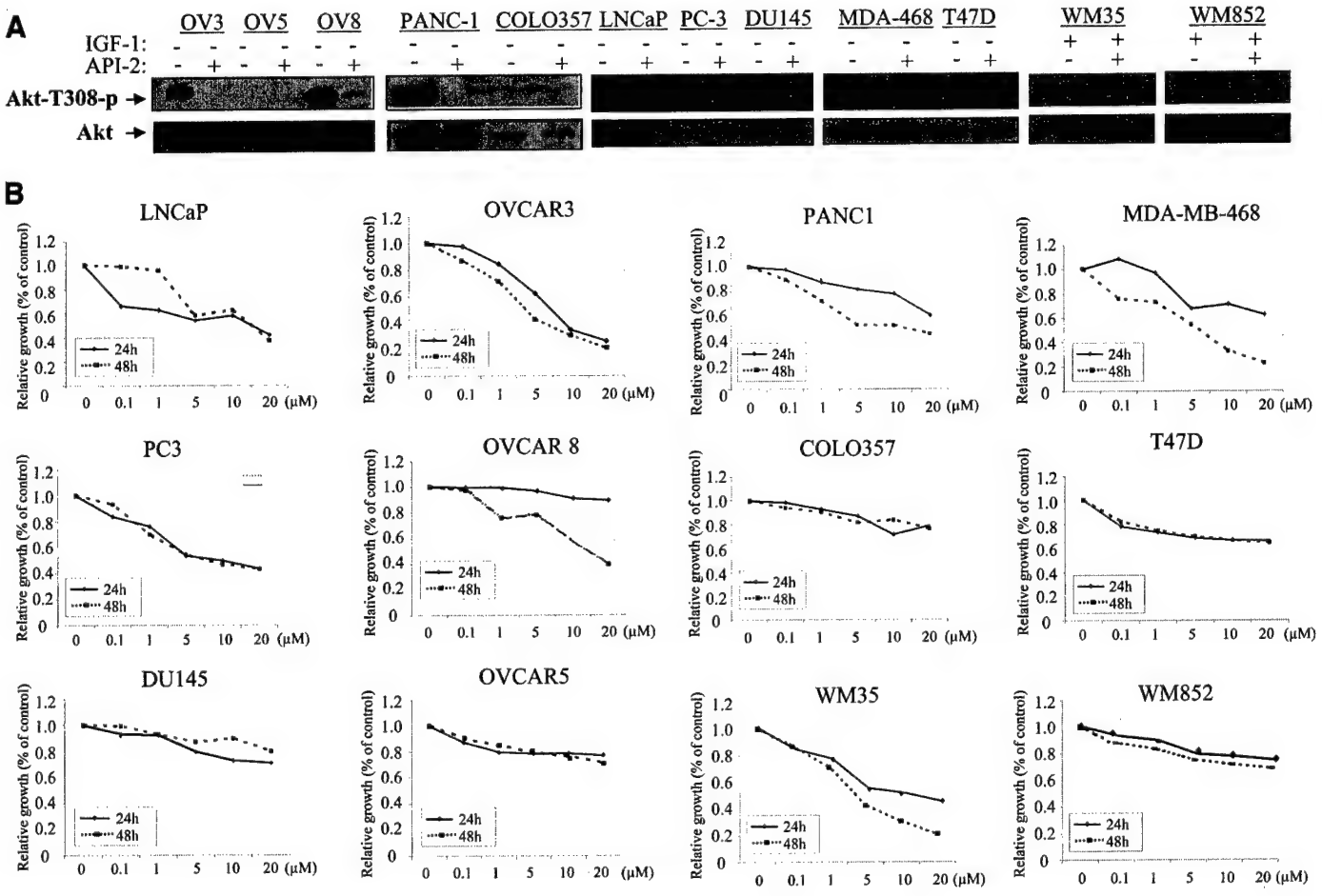

Fig. 3.

PKC, SGK, p90 ribosomal S6 kinase, $p70^{S6K}$, mitogen- and stress-activated protein kinase, and PKC-related kinase. Among AGC kinase family members, protein structures of PKA, PKC, and SGK are more close to Akt kinase than other members. Therefore, we next examined the effects of API-2 on the enzymatic activities of these three kinases. HEK293 cells were transfected with HA-tagged PKA, PKCα, or SGK. *In vitro* kinase assay and immunoblotting analysis showed that the kinase activities of PKA and PKCα were inhibited by PKAI and Ro 31-8220, a PKC inhibitor, respectively, whereas API-2 exhibited no effect on their activities (Fig. 2, *C* and *E*). Furthermore, EGF-induced SGK kinase activity was attenuated by wortmannin but not by API-2 (Fig. 2*D*). In addition, we determined whether API-2 has effect on other oncogenic survival pathways. Western blotting analyses with commercially available anti-phospho-antibodies revealed that phosphorylation levels of signal transducer and activators of transcription 3, c-Jun NH_2-terminal kinase, p38, and extracellular signal-regulated kinase-1/2 were not affected by API-2 treatment (Fig. 2*F*). These data indicate that API-2 specifically inhibits Akt signaling pathway.

API-2 Suppresses Cell Growth and Induces Apoptosis in Akt-Overexpressing/Activating Human Cancer Cell Lines. The ability of API-2 to selectively inhibit the Akt pathway suggests that it should inhibit proliferation and/or induces apoptosis preferentially in those tumor cells with aberrant expression/activation of Akt. Because activation of Akt in human malignancies commonly results from overexpression of Akt or *PTEN* mutations, API-2 was used to treat the cells that express constitutively active Akt, caused by overexpression of AKT2 (OVCAR3, OVCAR8, PANC1, and AKT2-transformed NIH3T3) or mutations of the *PTEN* gene (PC-3, LNCaP, and MDA-MB-468), and cells that do not (OVCAR5, DU-145, T47D, COLO357, and LXSN-NIH3T3), as well as melanoma cells that are activated by insulin-like growth factor-I to activate Akt or do not respond to growth stimulation by insulin-like growth factor-I (6). Immunoblotting analysis showed that phosphorylation levels of Akt were inhibited by API-2 only in the cells expressing elevated Akt or responding to insulin-like growth factor-I simulation (Fig. 3*A*). Accordingly, API-2 inhibited cell growth to a much higher degree in Akt-overexpressing/activating cells compared with those with low levels of Akt. As shown in Fig. 3*B*, API-2 treatment inhibited cell proliferation by approximately 50–60% in Akt-overexpressing/activating cell lines, LNCaP, PC-3, OVCAR3, OVCA8, PANC1, MDA-MB-468, and WM35, whereas only by about 10–20% in DU145, OVCAR5, COLO357, T47D, and WM852 cells, which exhibit low levels of Akt or do not respond to growth stimulation by insulin-like growth factor-I. Moreover, API-2 induces apoptosis by 8-fold (OVCAR3), 6-fold (OVCAR8), 6-fold (PANC1), and 3-fold (AKT2-NIH3T3). No significant difference of apoptosis was observed between API-2 and vehicle (DMSO) treatment in OVCAR5, COLO357, and LXSN-NIH3T3 cells (Fig. 3*C*). Thus, API-2 inhibits cell growth and induces apoptosis preferentially in cells that express aberrant Akt.

API-2 Inhibits Downstream Targets of Akt. It has been shown that Akt exerts its cellular effects through phosphorylation of a number of proteins (1). More than 20 proteins have been identified as Akt substrates, including the members of Forkhead protein family (FKHR, AFX, and FKHRL1), tuberlin/TSC2, $p70^{S6K}$, GSK-3β, $p21^{WAF1/Cip1}$, $p27^{kip1}$, MDM2, Bad, ASK1, and IKKα, etc. We next examined whether API-2 inhibits downstream targets of Akt. Because anti-phospho-tuberlin, anti-phospho-Bad, anti-phospho-AFX, and anti-phospho-GSK-3β antibodies are commercially available, we therefore determined the effect of API-2 on their phosphorylation induced by

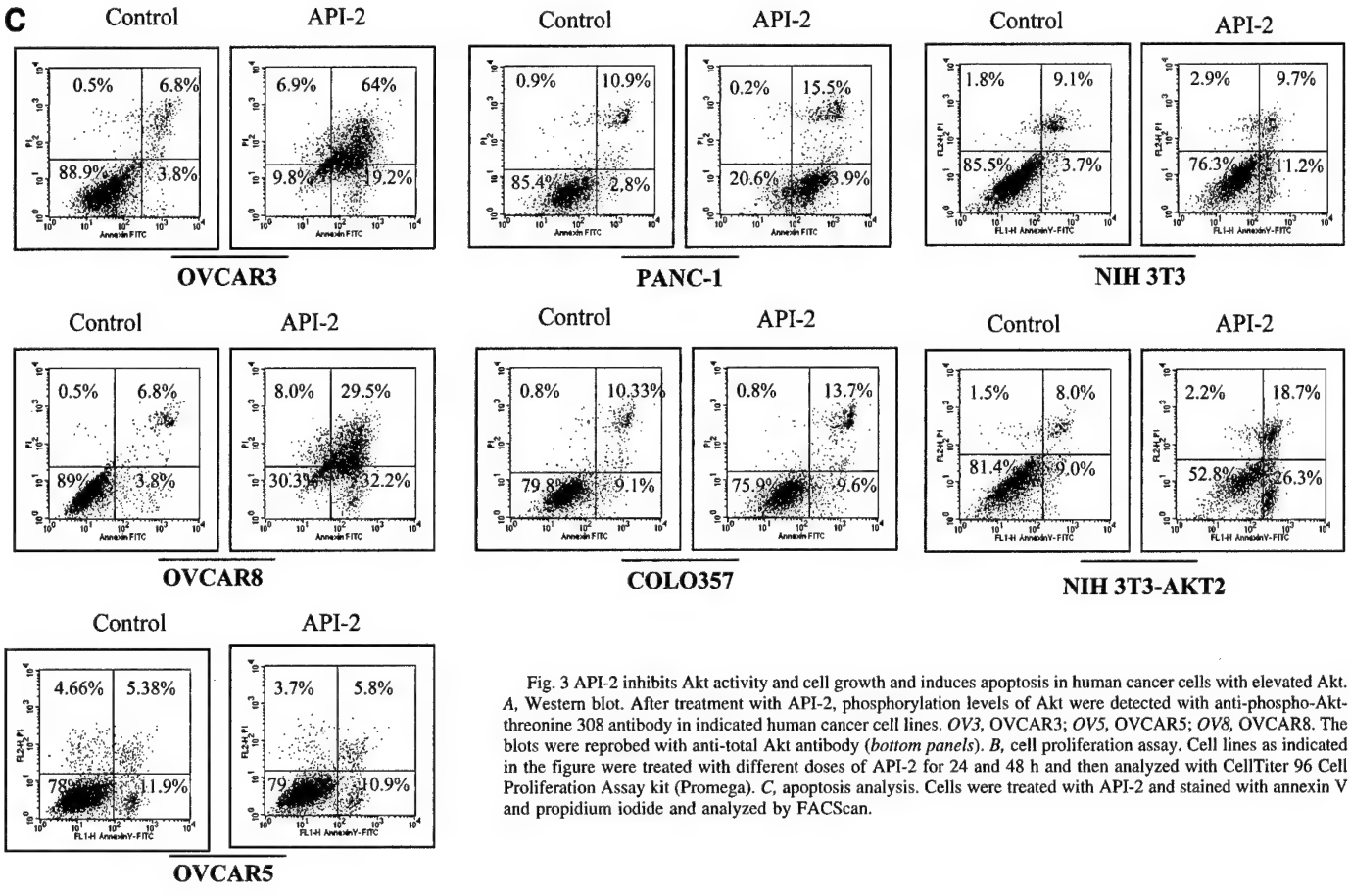

Fig. 3 API-2 inhibits Akt activity and cell growth and induces apoptosis in human cancer cells with elevated Akt. *A*, Western blot. After treatment with API-2, phosphorylation levels of Akt were detected with anti-phospho-Akt-threonine 308 antibody in indicated human cancer cell lines. *OV3*, OVCAR3; *OV5*, OVCAR5; *OV8*, OVCAR8. The blots were reprobed with anti-total Akt antibody (*bottom panels*). *B*, cell proliferation assay. Cell lines as indicated in the figure were treated with different doses of API-2 for 24 and 48 h and then analyzed with CellTiter 96 Cell Proliferation Assay kit (Promega). *C*, apoptosis analysis. Cells were treated with API-2 and stained with annexin V and propidium iodide and analyzed by FACScan.

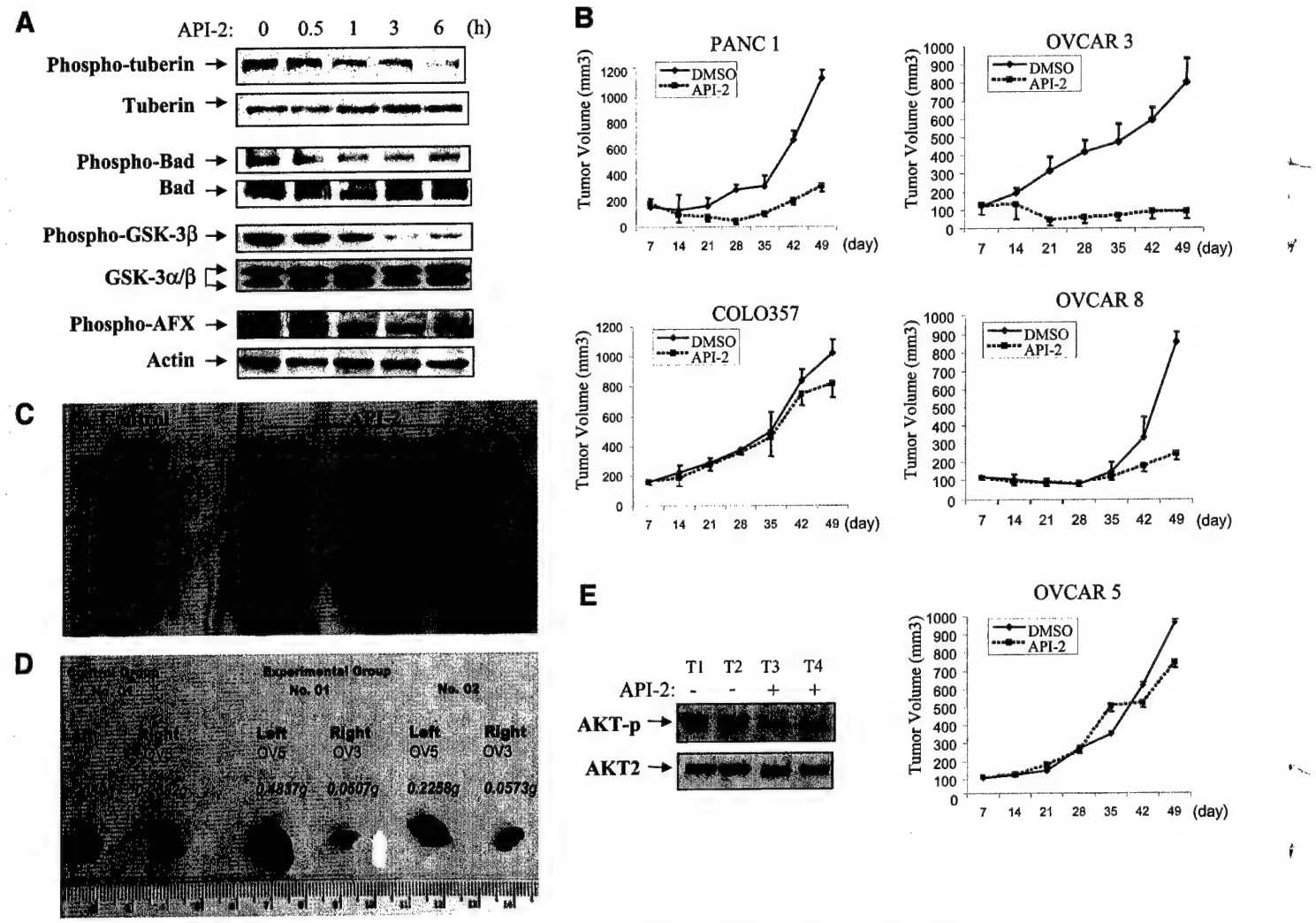

Fig. 4. API-2 inhibits downstream targets of Akt and exhibits antitumor activity in cancer cell lines with elevated Akt in mouse xenograft. *A*, API-2 inhibits Akt phosphorylation of tuberin, Bad, AFX, and GSK-3β. After treatment with API-2, OVAR3 cells were lysed and immunoblotted with indicated antibodies. *B*, API-2 inhibits tumor growth. Tumor cells were s.c. injected into nude mice with low level of Akt cells on left side and elevated level of Akt cells on right side. When the tumors reached an average size of about 100–150 mm^3, animals were treated with either vehicle or 1 mg/kg/day API-2 as described in "Materials and Methods." Each measurement represents an average of 10 tumors. *C*, representation of the mice with OVCAR3 (*right*) and OVCAR5 (*left*) xenograft treated with API-2 or vehicle (*control*). *D*, examples of tumor size (*bottom*) and weight (*top*) at the end of experiment. *E*, immunoblotting analysis of tumor lysates with anti-phospho-Akt-serine 473 (*top*) and anti-AKT2 (*bottom*) antibodies in OVCAR3-derived tumors that were treated (*T3* and *T4*) and untreated (*T1* and *T2*) with API-2.

Akt. After API-2 (1 μM) treatment, OVCAR3 cells were lysed and immunoblotted with the individual anti-phospho-antibody. Fig. 4*A* shows that API-2 considerably inhibited the phosphorylation levels of tuberlin leading to stabilization and up-regulation of tuberin (11). The phosphorylation levels of Bad, GSK-3β, and AFX were partially attenuated by API-2. These data suggest that API-2 induces cell death and cell growth arrest by inhibiting phosphorylation of its downstream targets. API-2 inhibition of Akt downstream targets at different degrees could be due to the fact that phosphorylation sites of these targets are also regulated by other kinase(s), for instance, Bad serine 136 is phosphorylated by PAK1 in addition to Akt (12).

API-2 Inhibits the Growth of Tumors in Nude Mice That Overexpress Akt. We have previously shown frequent overexpression/activation and/or amplification of AKT1 and AKT2 in human ovarian and pancreatic cancer (2). Inhibition of Akt pathway by inhibitors of PI3k, HSP70, Src, and farnesyltransferase resulted in cell growth arrest and induction of apoptosis (13, 14). A recent study showed that the tumor growth of xenografts with elevated Akt was also significantly inhibited by intratumoral injection of adenovirus of dominant-negative Akt (9). Because API-2 inhibits Akt signaling and induces apoptosis and cell growth arrest only in cancer cells with elevated levels of Akt (Fig. 3), we reasoned that the growth of tumors with elevated levels of Akt should be more sensitive to API-2 than that of tumors with low levels of Akt in nude mice. To this end, we s.c. implanted Akt-overexpressing cells (OVCAR3, OVCAR8, and PANC-1) into the right flank and those cell lines that express low levels of Akt (OVCAR5 and COLO357) into the left flank of mice. When the tumors reached an average size of about 100–150 mm^3, the animals were randomized and treated i.p. with either vehicle or API-2 (1 mg/kg/day). As illustrated in Fig. 4*B*, OVCAR5 and COLO357 tumors treated with vehicle grew to about 800–1,000 mm^3 49 days after tumor implantation. OVCAR3, OVCAR8, and PANC1 tumors treated with vehicle control grew to about 700–900 mm^3 49 days after tumor implantation. API-2 inhibited OVCAR3, OVCAR8, and PANC1 tumor growth by 90, 88, and 80%, respectively. In contrast, API-2 had little effect on the growth of OVCAR5 and COLO357 cells in nude mice (Fig. 4, *B–D;* data not shown). At a dose of 1 mg/kg/day, API-2 had no effect on blood glucose level, body weight, activity, and food intake of mice. In treated tumor samples, Akt activity was inhibited by API-2 without a change of total Akt content (Fig. 4*E*). Taken together, these results indicate that API-2 selectively inhibits the growth of tumors with elevated levels of Akt.

Discussion

In this study, we identified a small molecule inhibitor of Akt signaling, API-2, by screening the NCI diversity set. Treatment of human cancer cells with API-2 suppresses Akt signaling without obvious inhibitory effects on a number of other oncogenic kinases examined. In tumor xenograft mouse model, we further demonstrated that the antitumor effect of API-2 is selective for those tumors with aberrant Akt. Significantly, the high degree of efficacy was achieved at a low dose of API-2 and no compound related side effects were observed. In contrast, little efficacy was seen with tumors displaying low levels of Akt. API-2 is a synthetic small molecule compound identified previously and named TCN or tricyclic nucleoside (10). Previous studies have shown that API-2/TCN inhibits DNA synthesis and has antitumor and antiviral activity (15, 16). Our data indicate that API-2/TCN inhibition of Akt pathway plays a key role in its antitumor activity.

Phase I and II clinic trails of API-2/TCN have been conducted on advanced tumors (17, 18). API-2/TCN exhibited some side effects, which include hepatotoxicity, hypertriglyceridemia, thrombocytopenia, and hyperglycemia (17, 18). It is not clear whether the hyperglycemic effect of API–2/TCN relates to the inhibition of Akt activation. Recent knock-out mouse studies have shown that the mice deficient in *Akt2* are impaired in the ability of insulin to lower blood glucose because of defects in the action of insulin on skeletal muscle and liver. $Akt2^{-/-}$ mice are born without apparent defects but develop peripheral insulin resistance and nonsuppressible hepatic glucose production, resulting in hyperglycemia accompanied by inadequate compensatory hyperinsulinemia (19). In contrast, *Akt1*-difficient mice did not display a diabetic phenotype (20). The mice are viable but display impairment in organismal growth. Such relatively subtle phenotypic change in $Akt1^{-/-}$ mice suggests that Akt2 and Akt3 may substitute to some extent for Akt1 (20). Although a high dose of API-2/TCN-induced hyperglycemia may be due to inhibition of AKT2 activation in human, the compound-exhibited potent stimulation of apoptosis and inhibition of tumor cell growth must result from inhibition of all three isoforms of Akt. The side effects of API-2/TCN have been shown to closely relate to the dose in the clinic trails (17, 18). Due to its severe side effects at high doses, API-2/TCN has been limited in the clinic. In this study, we demonstrated that low dose of API-2 effectively and selectively induces apoptosis and inhibits growth in tumor cells with elevated levels of Akt. In xenograft experiments, no visible side effects were observed in 50 mice treated with API-2 at concentration of 1 mg/kg/day, which significantly inhibited tumor growth in Akt-overexpressing cancer cells. These data indicate that API-2 at low doses could achieve antitumor growth without significant side effect in tumors with elevated Akt. Therefore, protocols for additional assessment of API-2/TCN in the clinic must incorporate careful patient selection based on Akt status in the tumor.

In summary, we have demonstrated that API-2/TCN is a potent and selective inhibitor of Akt signaling pathway in tumor cells. API-2 blocks Akt pathway, leading to the induction of apoptosis and cell growth arrest. Additional studies are required to elucidate the mechanism by which API-2/TCN blocks Akt activation. The ability of API-2 to inhibit growth of human tumor xenografts in nude mice provides validation for the development of drugs targeting Akt to treat cancers displaying elevated levels of Akt. Additional investigation is required to evaluate whether API-2/TCN is clinically useful in this setting.

Acknowledgments

We thank Drs. Edward Sausville, Jill Johnson, George Johnson, Daniel Zaharevitz, and Robert Schultz from the NCI Developmental Therapeutics Program for providing us with the Diversity Set.

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Appendix 2

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 7, Issue of February 13, pp. 5405–5412, 2004
Printed in U.S.A.

Akt Phosphorylation and Stabilization of X-linked Inhibitor of Apoptosis Protein (XIAP)*

Received for publication, November 3, 2003, and in revised form, November 25, 2003
Published, JBC Papers in Press, November 25, 2003, DOI 10.1074/jbc.M312044200

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Akt negatively regulates apoptotic pathways at a pre-mitochondrial level through phosphorylation and modulation of proteins such as Bad, Forkhead proteins, and GSK-3β. Akt has also been shown to protect cell death at a post-mitochondrial level, although its downstream targets have not been well documented. Here, we demonstrate that Akt, including AKT1 and AKT2, interacts with and phosphorylates X-linked inhibitor of apoptosis protein (XIAP) at residue serine-87 *in vitro* and *in vivo*. Phosphorylation of XIAP by Akt protects XIAP from ubiquitination and degradation in response to cisplatin. Moreover, autoubiquitination of XIAP is also inhibited by Akt. Consistent with this, an XIAP mutant introduced into cells which mimics the Akt-phosphorylated form (*i.e.* XIAP-S87D) displays reduced ubiquitination and degradation as compared with wild type XIAP. The greater stability of XIAP-S87D in cells translated to increased cell survival after cisplatin treatment. Conversely, a mutant that could not be phosphorylated by Akt (XIAP-S87A) was more rapidly degraded and showed increased cisplatin-induced apoptosis. Furthermore, suppression of XIAP by either siRNA or adenovirus of antisense of XIAP induced programmed cell death and inhibited Akt-stimulated cell survival in ovarian cancer cells. These data identify XIAP as a new downstream target of Akt and a potentially important mediator of the effect of Akt on cell survival.

Akt, also named protein kinase B (PKB)[1] or RAC kinase, is a family of phosphatidylinositol 3-OH-kinase-regulated serine/threonine kinase (1–3). Three isoforms of Akt have been identified: Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ, all of which are activated by growth factors in a phosphatidylinositol 3-OH-kinase-dependent manner (4–6). Full activation of the Akts requires their phosphorylation at Thr^{308} (Akt1), Thr^{309} (Akt2), or Thr^{305} (Akt3) in the activation loop and Ser^{473} (Akt1), Ser^{474} (Akt2), or Ser^{472} (Akt3) in the C-terminal activation domain (7).

Accumulated evidence shows that Akt and its downstream targets constitute a major cell survival pathway. Akt promotes cell survival and suppresses apoptotic death in a number of cell types induced by a variety of stimuli, including growth factor withdrawal, cell cycle discordance, and loss of cell adhesion (7). Several downstream targets containing the Akt phosphorylation consensus sequence (R-*X*-R-*X*-*X*-S/T) have been identified which shed light on the mechanisms by which Akt promotes cell survival and blocks apoptosis. The first anti-apoptotic Akt target identified was the pro-apoptotic protein BAD. BAD is a pro-death member of the Bcl-2 family that initiates apoptosis by binding to Bcl-$x_L$on the outer mitochondrial membrane, causing the release of cytochrome *c* into the cytosol. Akt phosphorylates BAD on Ser^{136}, promoting the association of BAD with 14-3-3 proteins in the cytosol and inactivating its pro-apoptotic function (7). The execution of cellular apoptosis also involves changes in the transcriptional program (7). Akt decreases the transcription of a subset of death genes by phosphorylation of the Forkhead family of transcription factors, which causes their nuclear exclusion and inactivation (7). Akt also phosphorylates and activates the cyclic AMP-response element-binding protein, which increases the transcription of anti-apoptotic genes, such as Bcl-2 (8, 9). In addition, recent studies have shown that Akt phosphorylates and inactivates apoptosis signal-regulating kinase-1, a mitogen-activated protein kinase kinase kinase that mediates stress- and cytokine-induced cell death (10, 11). Akt also phosphorylates the murine double minute-2 protein promoting its translocation to the nucleus and the destabilization of p53 (12). By promoting the degradation of p53, Akt impairs the cellular stress response, increasing the survival of tumor cells. A role for an anti-apoptotic function of Akt at a post-mitochondrial step has also been proposed (13), and Akt has been reported to directly phosphorylate and inactivate the cell death protease caspase-9 (14). In addition, Akt has also been shown to inhibit the activation of Bax during apoptosis through an uncharacterized mechanism (11, 15).

The inhibitor of apoptosis proteins (IAPs) are a family of intracellular anti-apoptotic proteins, first identified in baculovirus, which play a key role in cell survival by modulating death-signaling pathways at a post-mitochondrial level. They currently include X-linked IAP (XIAP), human IAP-1 (Hiap-1),

* This work was supported in part by NCI National Institutes of Health Grants CA77935 and CA89242, Department of Defense Grants DAMD 17-01-1-0394 and DAMD 17-02-1-0670, Canadian Institutes of Health Research Grant MOP-15691, and National Cancer Institute of Canada Grant 013335 (with funds from the Canadian Cancer Society). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PKB, protein kinase B; XIAP, X-linked inhibitor of apoptosis protein; IAP, inhibitor of apoptosis protein; HA, hemagglutinin; HEK, human embryonic kidney; GST, glutathione *S*-transferase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; siRNA, short interference RNA; E3, ubiquitin protease ligase; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone.

human IAP-2 (Hiap-2), neuronal apoptosis inhibitory protein (Naip), Survivin, and Livin. These proteins are characterized by the presence of a caspase-recruitment domain and an N-terminal baculovirus-inhibitor-of-apoptosis-repeat motif, which is necessary for biological activity. With the exception of Naip and survivin, IAPs also contain a C-terminal RING-zinc finger domain believed to be involved in protein-protein and protein-nucleic acid interactions (16). Recent studies have shown that the RING finger domain has ubiquitin protease ligase (E3) activity and is responsible for the autoubiquitination and degradation of IAPs after an apoptosis stimulus (17). Among human IAPs, XIAP is the most potent inhibitor of caspases and apoptosis. It has been shown that XIAP is a direct inhibitor of caspase-3 and caspase-9 and modulates the Bax/cytochrome *c* pathway by inhibiting caspase-9 (18).

In the present report, we demonstrate that XIAP is a physiological substrate of Akt. Akt interacts with and phosphorylates XIAP at serine 87. Phosphorylation of XIAP by Akt inhibits both its autoubiquitination and cisplatin-induced ubiquitination. These effects reduce XIAP degradation and the increased levels of XIAP are associated with decreased cisplatin-stimulated caspase 3 activity and programmed cell death.

MATERIALS AND METHODS

Cell Lines and Transfection—The human ovarian cancer epithelial cell line A2780S and human embryonic kidney (HEK) 293 cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The cells were transfected with appropriate DNA indicated in the figure legends, using LipofectAMINE Plus (Invitrogen).

Plasmid Constructs—Akt plasmids have previously been described (19, 20). FLAG-tagged XIAP was generated by reverse transcriptase-PCR and subcloned to p3XFLAG-CMV-10 vector (Sigma). XIAP-S87A and XIAP-S87D mutant constructs were created using a QuikChange site-directed mutagenesis kit (Stratagene). Myc-tagged ubiquitin and Flg-Bcl2 constructs was kindly provided by Drs. Wenlong Bai and Gen Wang. The glutathione *S*-transferase (GST)-XIAP and GST-XIAPS87A were created by PCR and subcloned to pGEX-4T1.

Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End Labeling (TUNEL) and Caspase-3 Activity Assays—Apoptotic cells were detected with TUNEL using an *in situ* cell death detection kit (Roche Applied Science). For caspase-3 activity, following cisplatin treatment, cells were harvested, lysed, and assayed with caspase-3 kit.

GST Fusion Protein, Immunoprecipitation, Immunoblotting, and in Vitro Kinase Assay—GST-XIAP fusion proteins, immunoprecipitation, and immunoblotting were performed as described previously (19, 20). For *in vitro* kinase assay, Akt immunoprecipitates were incubated with a kinase buffer (19). GST-XIAP fusion proteins were used as the exogenous substrate. Each experiment was repeated three times.

In Vivo [^{32}P]Orthophosphate Cell Labeling and Pulse-Chase Experiments—COS7 cells were transfected with FLAG-XIAP together with or without constitutively active Akt and labeled with [^{32}P]orthophosphate (0.5 mCi/ml) in minimum Eagle's medium without phosphate for 2 h. FLAG-XIAP was immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were separated on SDS-PAGE and transferred to membranes. Phosphorylated XIAP was detected by autoradiography. Pulse-chase was performed as described previously (20).

RESULTS

Akt Stabilizes XIAP—XIAP is a mammalian prototype of the IAP family and suppresses the programmed cell death by direct inhibition of caspase-9 and caspase-3 activity (16). Down-regulation of XIAP is an important mechanism for caspase activation in response to different apoptotic stimuli, including chemotherapeutic agents (21). We and others (22, 23) have previously shown that cisplatin-induced programmed cell death is accompanied by a decrease in XIAP protein content. We also found that activation of the Akt pathway induces cisplatin resistance by inhibition of apoptosis (24). Together, these findings prompted us to examine whether Akt inhibits cisplatin-induced XIAP degradation. A2780S ovarian cancer cells, which are sensitive to cisplatin, were stably transfected with constitutively active AKT2 (Myr-AKT2) or pcDNA3 vector alone (Fig. 1*A*). In pcDNA3-transfected cells, cisplatin (10 and 20 μM)) treatment induces a significant decline of XIAP protein expression. In contrast, XIAP levels were stable in Myr-AKT2-transfected A2780S cells (Fig. 1, *B* and *C*). TUNEL assay analysis showed that Myr-AKT2 also protected A2780S cells from cisplatin-induced apoptois (Fig. 1*C*). In addition, cisplatin-induced down-regulation of XIAP is cisplatin-dose-dependent (Fig. 1, *B* and *C*). To determine whether Akt effects XIAP at the transcriptional level, we performed Northern blot analysis using total RNA prepared from pcDNA3-A2780S or Myr-AKT2-transfected A2780S cells following treatment with cisplatin. As shown in Fig. 1*D*, levels of XIAP mRNA were similar in both cell lines and not effected by cisplatin, ruling out transcriptional regulation of XIAP by cisplatin and Akt. A second possibility is that XIAP expression and its down-regulation by cisplatin treatment of cells is predominantly regulated post-translationally via a protein degradation mechanism. To test this hypothesis, we performed a pulse-chase assay. pcDNA3-A2780S and Myr-AKT2-A2780S cells were metabolically labeled with [^{35}S]methionine for 1 h. Following change of medium, immunoprecipitation was carried out with anti-XIAP antibody at 0, 2, and 4 h. The amount of labeled XIAP was visualized by SDS-PAGE and autoradiography of the gels. As shown in Fig. 1*E*, XIAP was almost completely degraded within 4 h in pcDNA3-A2780S cells, whereas the degradation rate was significantly lower in the cells expressing constitutively active AKT2. These data suggest that Akt promotes increased levels of XIAP in A2780S cells by protecting the protein from degradation.

Because the members of IAP family are transcriptionally regulated by NFκB pathway (16) and ectopic expression of Akt activates NFκB cascade (25, 26), we further examined whether Akt induces XIAP translation and/or transcription. HEK293 cells were transfected with increasing amount of constitutively active Akt. Western and Northern blot analyses showed that ectopic expression of constitutively active Akt did not induce XIAP at protein and mRNA levels (Fig. 2, *A* and *B*), suggesting that Akt rescuing cisplatin-down-regulated XIAP is not resulted from up-regulation of XIAP at translational/transcriptional level.

Recent reports have demonstrated that mitochondria released Omi/HtrA2 cleavages IAP family proteins, including c-IAP1 and XIAP, during the apoptosis (27, 28). As cisplatin induces programmed cell death via mitochondrial pathway (11, 22), we next examined the possible involvement of Omi in cisplatin-induced XIAP degradation. A2780S cells were stably transfected with Bcl2, which is known to stabilize mitochondria in response to cisplatin treatment (29). The transfected cells were treated with cisplatin for 12 h. Immunoblotting and TUNEL assay analyses showed that unlike constitutively active Akt, Bcl2 only protected XIAP from cisplatin-induced down-regulation at about 20% even though it significantly rescued cisplatin-caused apoptosis (Fig. 2*C*). A second possibility of cisplatin-induced XIAP degradation is via either caspase or proteasome pathway. To this end, A2780S cells were treated with cisplatin together with or without caspase inhibitor Z-VAD-fmk or proteasome inhibitor MG132 and/or lactacystin. As shown in Fig. 2*D*, the XIAP degradation was significantly inhibited by proteasome inhibitors but not by Z-VAD-fmk. These data indicate that the XIAP degradation induced by cisplatin is primarily through proteasome pathway.

Akt Inhibits Autoubiquitination and Cisplatin-induced Ubiquitination of XIAP—XIAP has been shown to possess E3 ubiquitin ligase activity, which is responsible for its autoubiquitination and ubiquitination in response to apoptotic stimuli (16, 21, 29). To examine whether Akt inhibits autoubiquitination of

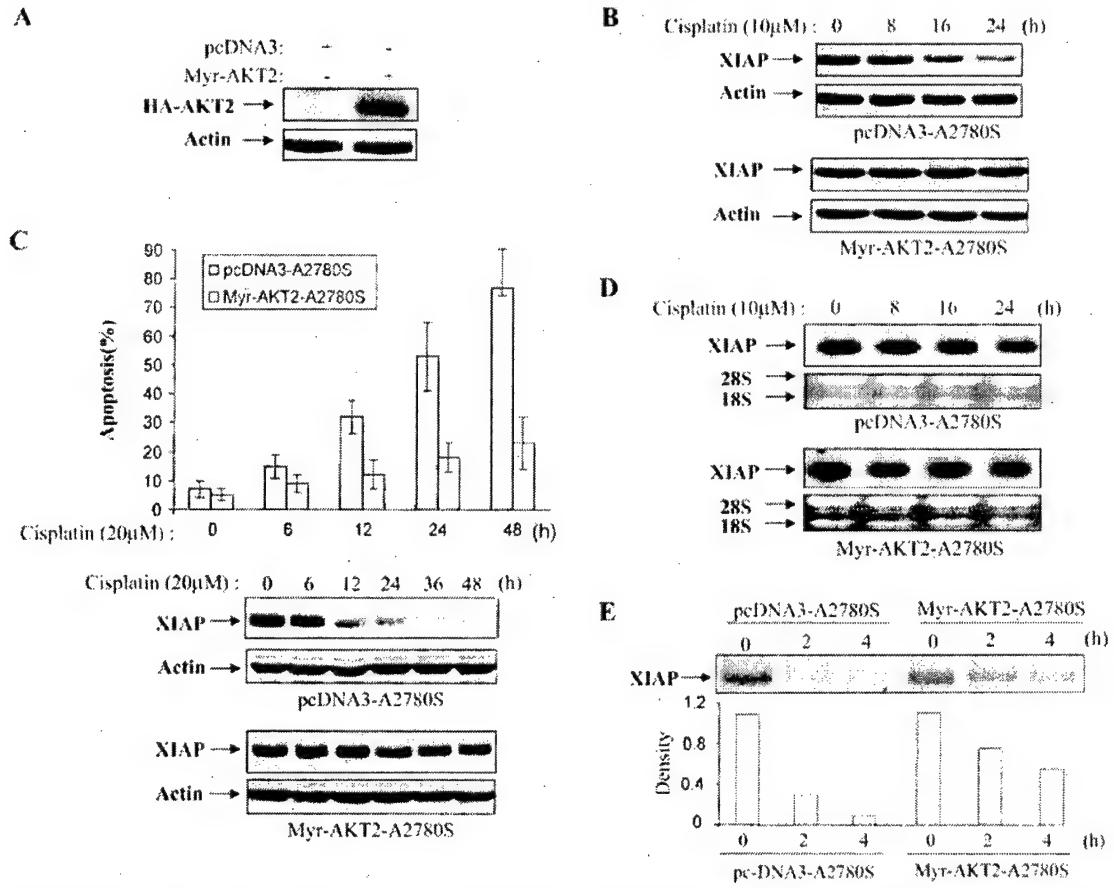

FIG. 1. **Constitutively active Akt inhibits cisplatin-induced XIAP down-regulation.** *A*, Western blotting analysis of human ovarian cancer A2780S cells stably transfected with HA-tagged constitutively active AKT2 (Myr-AKT2-A2780S) or pcDNA3 (pcDNA3-A2780S) with anti-HA (*top panel*) and anti-actin (*bottom panel*) antibodies. *B*, immunoblotting analysis. pcDNA3-A2780S (*top panels*) and Myr-AKT2-A2780S (*bottom panel*) cells were treated with cisplatin (10 μM) for the indicated times and lysed and then immunoblotted with the indicated antibodies. *C*, pcDNA3-A2780S and Myr-AKT2-A2780S cells were treated with cisplatin (20 μM) for indicated time. Apoptosis (*top*) was examined with TUNEL assay. Expression of XIAP and equal protein loading were detected with the indicated antibodies (*bottom panels*). *D*, Northern blot analysis. pcDNA3-A2780S and Myr-AKT2-A2780S cells were treated with cisplatin for indicated time. Total RNAs were isolated and subjected to Northern blot analysis with [^{32}P]dCTP-labeled XIAP cDNA probe (*first* and *third panels*). Equal loadings are indicated by 28 S and 18 S ribosomal RNA in the *second* and *fourth panels*. *E*, pulse-chase analysis. pcDNA3-A2780S and Myr-AKT2-A2780S cells were labeled with [^{35}S]methionine, chased at the indicated times and immunoprecipitated with anti-XIAP antibody. The immunoprecipitates were separated by SDS-PAGE, exposed to x-ray film (*top panel*), and quantified (*bottom panel*). Each experiment was repeated three times.

XIAP, HEK293 cells were transfected with Myc-tagged ubiquitin together with or without constitutively active Akt (Myr-Akt). Consistent with previous reports, ectopic expression of ubiquitin induces autoubiquitination of XIAP and its degradation in a manner sensitive to proteasome inhibitor MG132 (22, 29). Ectopic expression of constitutively active Akt significantly inhibited ubiquitin-induced autoubiquitination of XIAP (Fig. 3*A*) and prevented proteasome-mediated degradation of XIAP (Fig. 3*B*).

We next determined the effects of Akt on cisplatin-induced XIAP ubiquitination. HEK293 and A2780S cells were transfected with FLAG-XIAP and Myc-ubiquitin together with constitutively active or wild type Akt and then treated with cisplatin. Ubiquitination of XIAP was assayed by immunoprecipitation and immunoblotting after 12 h of the treatment. As shown in Fig. 3, *C* and *D*, cisplatin induces ubiquitination of XIAP that was significantly inhibited by expression wild type or/and constitutively active Akt. Taken together, we conclude that Akt stabilizes XIAP by inhibition of its autoubiquitination/ubiquitination.

Akt Phosphorylates and Interacts with XIAP—The fact that Akt regulates XIAP expression at a post-translation level suggests that XIAP may be a substrate of Akt. Indeed, protein sequence analysis revealed a consensus Akt phosphorylation sequence (R*X*R*XX*S/T) at residue serine 87 of XIAP, which is contained within the baculovirus-inhibitor-of-apoptosis-repeat 1 (BIR1) domain (Fig. 4*A*). To examine whether Akt phosphorylates XIAP, COS7 cells were co-transfected with FLAG-XIAP and constitutively active Akt1 and AKT2. After 36 h of transfection, cells were labeled with [^{32}P]orthophosphate for 3 h. Western blot analysis of FLAG-XIAP immunoprecipitates showed that both constitutively active Akt1 and AKT2 increase phosphorylation levels of XIAP as compared with cells transfected with XIAP and pcDNA3 vector (Fig. 4*B*). To examine whether Akt phosphorylates endogenous XIAP, HEK293 cells were either transfected with constitutively active AKT2 or serum-starved and then treated with LY294002 or vehicle (Me_2SO) prior to insulin stimulation. Endogenous XIAP was immunoprecipitated with anti-XIAP antibody, and the immunoprecipitates were subjected to immunoblotting analysis with anti-Akt substrate antibody. As shown in Fig. 4*B*, insulin and Akt2 induced phosphorylation of endogenous of XIAP. The phosphorylation induced by insulin was partially inhibited by phosphatidylinositol 3-OH-kinase inhibitor LY294002, suggesting that insulin also induce other kinase(s), in addition to Akt, to phosphorylate XIAP. To determine whether Akt phosphorylates XIAP at residue of sering-87, we generated GST

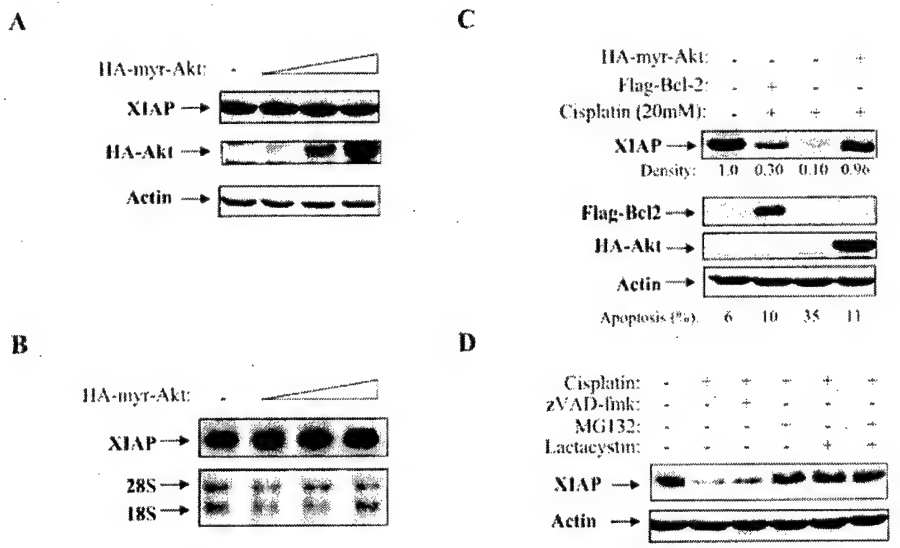

FIG. 2. **Akt does not regulate XIAP at transcriptional and translational levels; cisplatin down-regulates XIAP primarily through proteasome pathway.** Western (*A*) and Northern (*B*) blot analysis of expression of XIAP in HEK293 cells transfected with increasing amount of constitutively active Akt. The *top panels* show expression of XIAP protein (*A*) and mRNA (*B*). Expression of transfected HA-Myr-Akt was shown in *middle panel* of *A*. The *bottom panels* indicate equal loadings of protein (*A*) and RNA (*B*). *C*, Western blot and TUNEL assays. Stable FLAG-Bcl2- and HA-Myr-Akt-transfected A2780S cells were treated with cisplatin for 12 h and subjected to Western blot and TUNEL analysis. The *top panel* shows expression of XIAP. Quantification of XIAP protein levels is indicated below the *top panel*. Expression of transfected Bcl2 and Akt was detected with anti-FLAG (*second panel*) and -HA (*third panel*) antibodies. Actin was used as loading control (*fourth panel*). The *bottom panel* shows the percentage of apoptosis. *D*, Western blot analysis of XIAP expression in A2780S cells that were treated with cisplatin (20 μM), Z-VAD-fmk (20 μM), MG132 (25 μM), and/or lactacystin (25 μM) for 12 h (*top panel*). The *bottom panel* was detected with anti-actin antibody. All experiments were repeated three times.

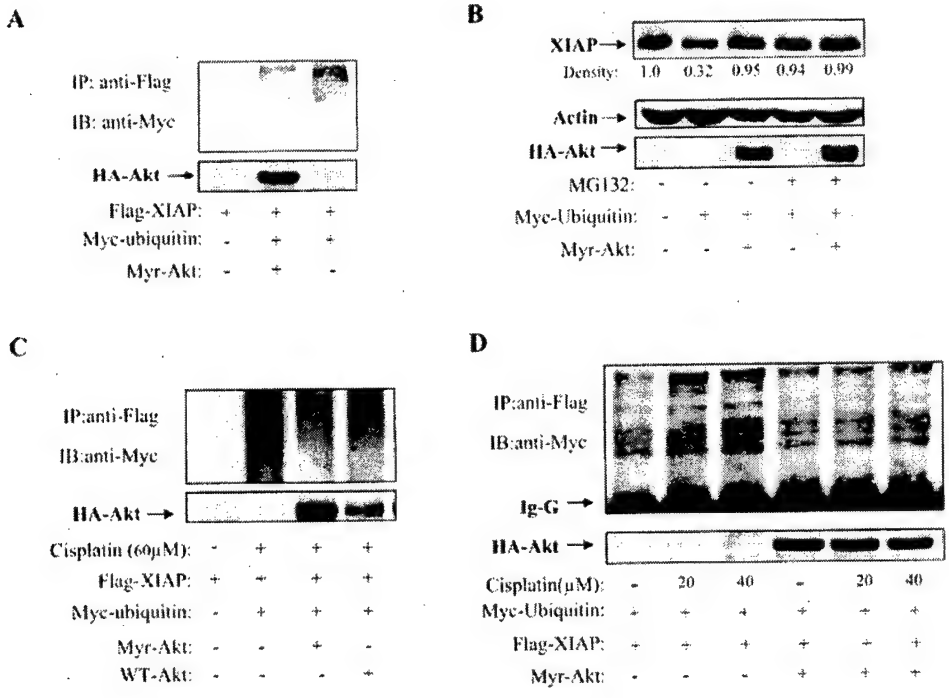

FIG. 3. **Ubiquitination and degradation of XIAP are reduced by expression of constitutively activated Akt.** *A*, Akt inhibits auto-ubiquitination of XIAP. HEK293 cells were transfected with indicated plasmids. After 48 h of transfection, cells were lysed and immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were immunoblotted with anti-Myc antibody (*top panel*). The *bottom panel* shows expression of transfected constitutively active Akt. *B*, constitutively active Akt and proteasome inhibitor suppress XIAP degradation. HEK293 cells were transfected with Myc-ubiquitin and constitutively active Akt. After 36 h of transfection, cells were treated with or without MG132 (100 μM) proteasome inhibitor for 12 h and subjected to immunoblotting analysis with anti-XIAP antibody (*top panel*). Expression of transfected constitutively active Akt and equal loading are shown in the *second* and *third panels*. *C* and *D*, Akt reduces cisplatin-induced XIAP ubiquitination. HEK293 (*C*) and A2780S (*D*) cells were transfected with indicated plasmids. After 36 h of transfection, cells were treated with the indicated amount of cisplatin for 12 h and lysed. Immunoprecipitation was performed with anti-FLAG antibody and immunoblotted with anti-Myc antibody.

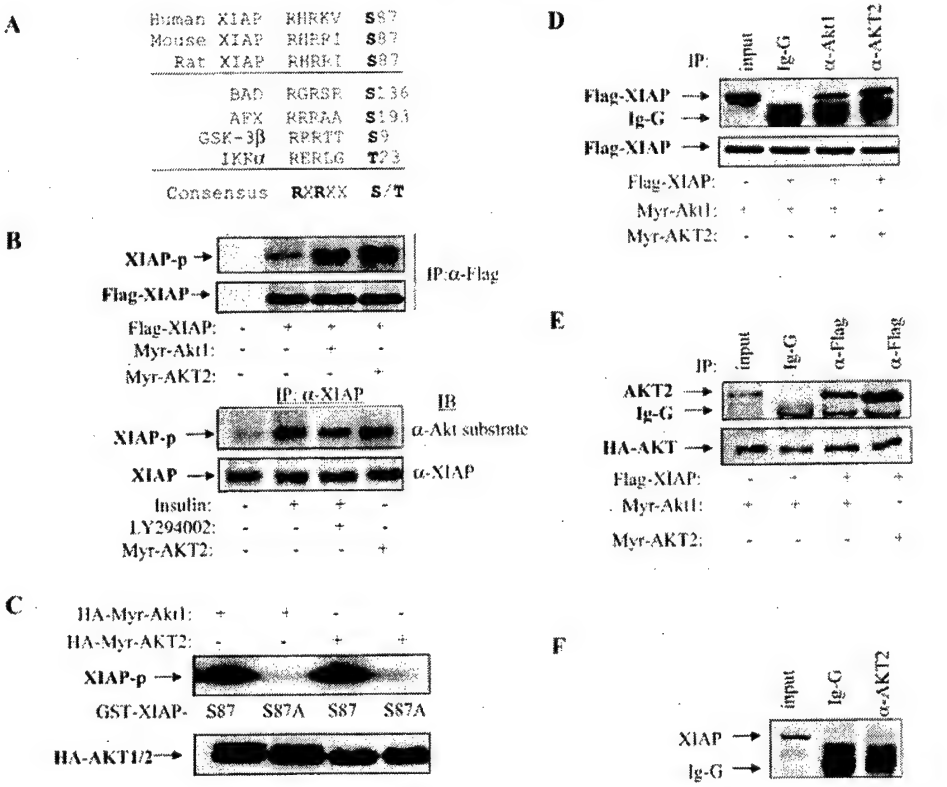

FIG. 4. **Akt phosphorylates and interacts with XIAP *in vitro* and *in vivo*.** *A*, a comparison of the putative Akt phosphorylation sites in human, mouse, and rat XIAP with the sequences of phosphorylation sites of several known Akt substrates. The phosphorylated residues are labeled by number, and a consensus sequence is denoted below. *B*, Akt1 and AKT2 phosphorylate XIAP *in vivo*. COS7 cells were transfected with indicated expression constructs, labeled with [^{32}P]orthophosphate, and immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were separated by SDS-PAGE, transferred to membrane, exposed to the film (*top panel*), and detected with anti-FLAG antibody (*second panel*). The *third* and *fourth panels* are Western blot analysis of the phosphorylation of endogenous XIAP in HEK293 cells. The cells were transfected and treated with the indicated plasmid and reagents and immunoprecipitated with anti-XIAP antibody. The immunoprecipitates were subjected to Western blot analysis with anti-Akt substrate (*third panel*) and anti-XIAP antibodies (*fourth panel*). *C*, Akt1 and AKT2 phosphorylate XIAP at residue serine 87 *in vitro*. *In vitro* kinase assay analysis of constitutively active Akt1 and AKT2 immunoprecipitates prepared from HEK293 cells transfected with HA-Myr-Akt1 and -AKT2 is shown. GST-fused wild type XIAP and XIAP-S87A proteins were used as substrates (*top panel*). Expression of transfected Akt1 and AKT2 is shown in the *bottom panel*. *D* and *E*, Akt interacts with XIAP. HEK293 cells were transfected with indicated plasmids. Following 48 h of incubation, cells were lysed, immunoprecipitated with anti-Akt1 or -AKT2 antibody, and detected with anti-FLAG antibody (*D*) or vice versa (*E*). Expression of transfected plasmids is shown in the *bottom panels*. *F*, endogenous AKT2 and XIAP associates with each other. HEK293 cells were lysed, immunoprecipitated with anti-AKT2, and detected with anti-XIAP antibody.

fusion proteins of wild type XIAP and XIAP-S87A, which was created by converting serine 87 residue into alanine. *In vitro* Akt kinase assay was carried out using the GST-XIAP and -XIAP-S87A fusion proteins as substrates. Repeated experiments revealed that Akt1 and AKT2 can efficiently phosphorylate wild type XIAP but not XIAP-S87A (Fig. 4*C*). These data indicate that XIAP is a potential physiological substrate of Akt kinase.

A number of Akt substrates have been shown to interact with Akt, which include IKKα, tuberin, and apoptosis signal-regulating kinase-1 (7, 10, 11). We therefore examined whether XIAP physically associates with Akt. HEK293 cells were co-transfected with HA-tagged Akt1, AKT2, and FLAG-XIAP. Immunoprecipitation was carried out with anti-HA and immunoblot of the immunoprecipitates was detected with anti-FLAG antibody or vice versa. As shown in Fig. 4, *D* and *E*, both Akt1 and AKT2 interact with XIAP. The binding affinity between AKT2 and XIAP is higher than that of Akt1 and XIAP. Furthermore, an endogenous protein-protein interaction between XIAP and Akt was also detected in HEK293 cells (Fig. 4*F* and data not shown).

Akt Inhibition of XIAP Ubiquitination/Degradation Depends on Phosphorylation—As demonstrated above, ectopic expression of Akt efficiently phosphorylates serine 87 of XIAP and inhibits XIAP ubiquitination and degradation. We next performed experiments to determine whether phosphorylation of XIAP at serine 87 accounts for the protective effect of Akt on XIAP ubiquitination/degradation. To test this hypothesis, Akt nonphosphorylatable (XIAP-S87A) and phosphomimic (XIAP-S87D) forms of XIAP were created and their capacity of autoubiquitination and cisplatin-induced ubiquitination was examined in HEK293 and A2780S cells, respectively. As shown in Fig. 5*A*, nonphosphorylatable XIAP-S87A and wild type XIAP underwent autoubiquitination when the cells were co-transfected with ubiquitin. Furthermore, autoubiquitination levels of phosphomimic XIAP-S87D decreased as compared with nonphosphorylatable and wild type XIAP. Unlike wild type XIAP (also seen in Fig. 3*B*), however, constitutively active Akt did not exhibit effects on either XIAP-S87A or XIAP-S87D autoubiquitination (Fig. 5*A*). Taken together, these results provide strong evidence that phosphorylation of XIAP at serine 87 occurs in cells, and this modification results in protection of XIAP from autoubiquitination.

To examine the impact of Akt phosphorylation of XIAP on cisplatin-induced ubiquitination and degradation of XIAP, A2780S cells were transfected with XIAP-S87A and XIAP-S87D. Following treatment with cisplatin for 12 h, we found that nonphosphorylatable XIAP-S87A was ubiquitinated in a

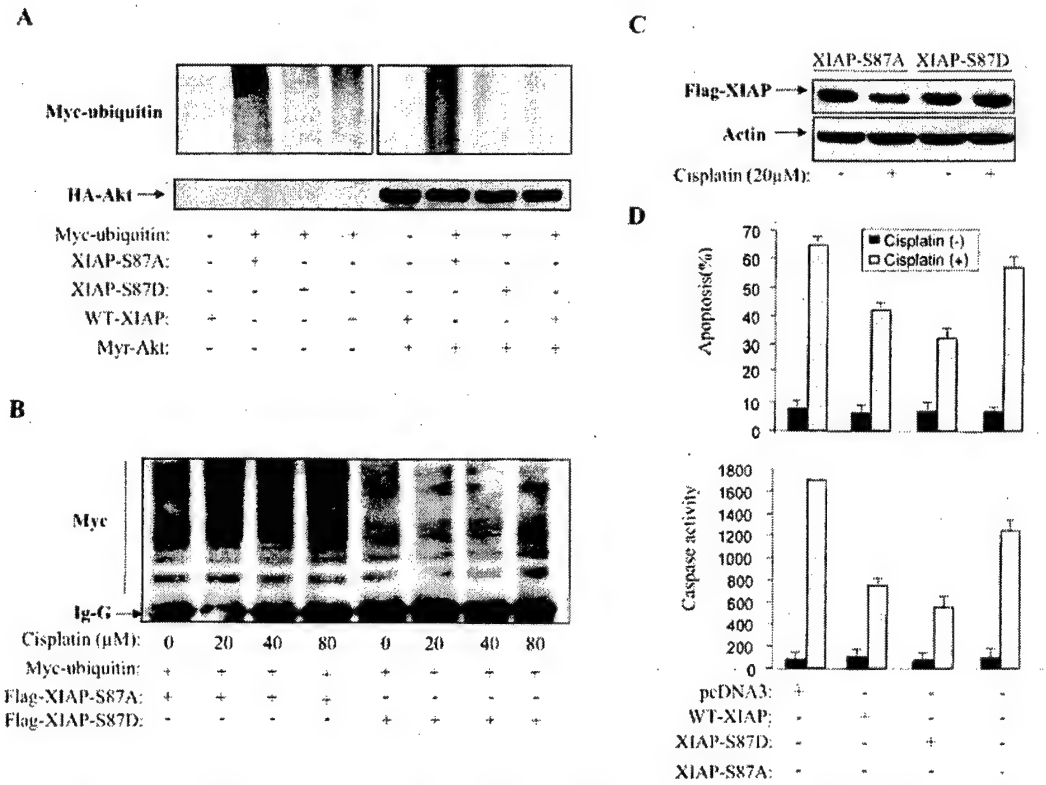

FIG. 5. **Akt phosphorylation of XIAP is required for Akt inhibition of XIAP ubiquitination and degradation.** *A*, the phosphorylation of XIAP inhibits its autoubiquitination. HEK293 cells were transfected with indicated plasmids. After 48 h of transfection, cells were lysed, immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-Myc antibody (*top panel*). The *bottom panel* shows expression of transfected Myr-Akt. *B*, cisplatin-induced XIAP ubiquitination decreased in its phosphomimic, but not nonphosphorylatable, form. A2780S cells were transfected with indicated plasmids, treated with different amounts of cisplatin for 12 h, lysed, and immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were immunoblotted with anti-Myc antibody. *C*, immunoblotting analysis of XIAP expression. A2780S cells were transfected with indicated plasmids, treated with cisplatin for 12 h, and immunoblotted with anti-FLAG antibody (*top panel*). Equal loading is shown in the *second panel*. *D*, TUNEL assay (*top panel*) and caspase-3 activity analysis (*bottom panel*). A2780S cells were transfected with indicated plasmids. After 36 h of transfection, cells were treated with or without cisplatin (20 μM) for 24 h. Apoptotic cells and capase-3 activity were examined by TUNEL assay and EnzChek caspase assay kit (Molecular Probes). The values represent three independent experiments.

cisplatin-dependent manner, whereas phosphomimic XIAP-S87D was resistant to cisplatin-induced ubiquitination (Fig. 5*B*). Accordingly, we also observed significantly reduced protein levels of nonphosphorylatable XIAP-S87A but not phosphomimic XIAP-S87D in response to cisplatin treatment (Fig. 5*C*). From these studies, we conclude that Akt inhibition of cisplatin-induced ubiquitination and degradation of XIAP are also done in a phosphorylation-dependent manner.

Effect of Akt Phosphorylation of XIAP on Its Antiapoptotic Function—As XIAP exhibits its anti-apoptotic effects by inhibiting caspase-3 and caspase-9, and our data show Akt inhibition of ubiquitination/degradation of XIAP by phosphorylation of XIAP, we next examined whether the phosphorylation of XIAP affects its antiapoptotic function. Wild type XIAP, nonphosphorylatable XIAP-S87A, and phosphomimic XIAP-S87D were introduced into A2780S cells. Following cisplatin treatment for 24 h, caspase-3 activity and apoptosis were analyzed. As shown in Fig. 5*D*, ectopic expression of all three forms of XIAP inhibits cisplatin-induced apoptosis and caspase-3 activity as compared with the cells transfected with pcDNA3 vector alone. However, nonphosphorylatable XIAP-S87A exhibited significantly lower antiapoptotic activity, whereas phosphomimic XIAP-S87D showed relatively higher anti-apoptotic function when compared with wild type XIAP.

To further determine the significance of Akt phosphorylation/stabilization of XIAP in Akt survival signaling, Myr-AKT2- and pcDNA3-A2780S cells were transfected or infected with siRNA or adenovirus of antisense of XIAP and then treated with cisplatin or vehicle (Me_2SO) for 12 h. Immunoblotting analysis showed that expression of XIAP was significantly inhibited by siRNA and antisense of XIAP (Fig. 6, *A* and *C*). The suppression of XIAP expression induces apoptosis in both AKT2- and pcDNA3-A2780S cells as compared with the cells treated with control siRNA or adenovirus of LacZ, which is consistent with recent reports in other cancer cells (30, 31). Furthermore, siRNA and adenovirus of antisense of XIAP largely abrogated constitutively active AKT2 protection of the cells from cisplatin-induced apoptosis (Fig. 6, *B* and *D*). These data indicate that XIAP is an important survival molecule and mediates Akt-induced cell survival and cisplatin resistance in A2780S cells.

DISCUSSION

XIAP is regulated at transcriptional and post-transcriptional levels. Activation of NFκB pathway induces RNA level of XIAP (16, 32). Since it possesses E3 ubiquitin ligase activity, XIAP can be autoubiquitinated and ubiquitinated in response to DNA damage, including treatment with chemotherapeutic agents (16, 21, 29). Recent studies showed that XIAP is cleaved by the mature serine protease Omi/HtrA2, a proapoptotic protein released from the mitochondria into the cytosol during apoptosis (27, 28). In this study, we demonstrate that the phosphorylation of XIAP by Akt regulates its ubiquitination and degradation. Akt, including Akt1 and AKT2, interacts with and phosphorylates XIAP, leading to inhibition of XIAP autoubiquitination and cisplatin-induced ubiquitination. We fur-

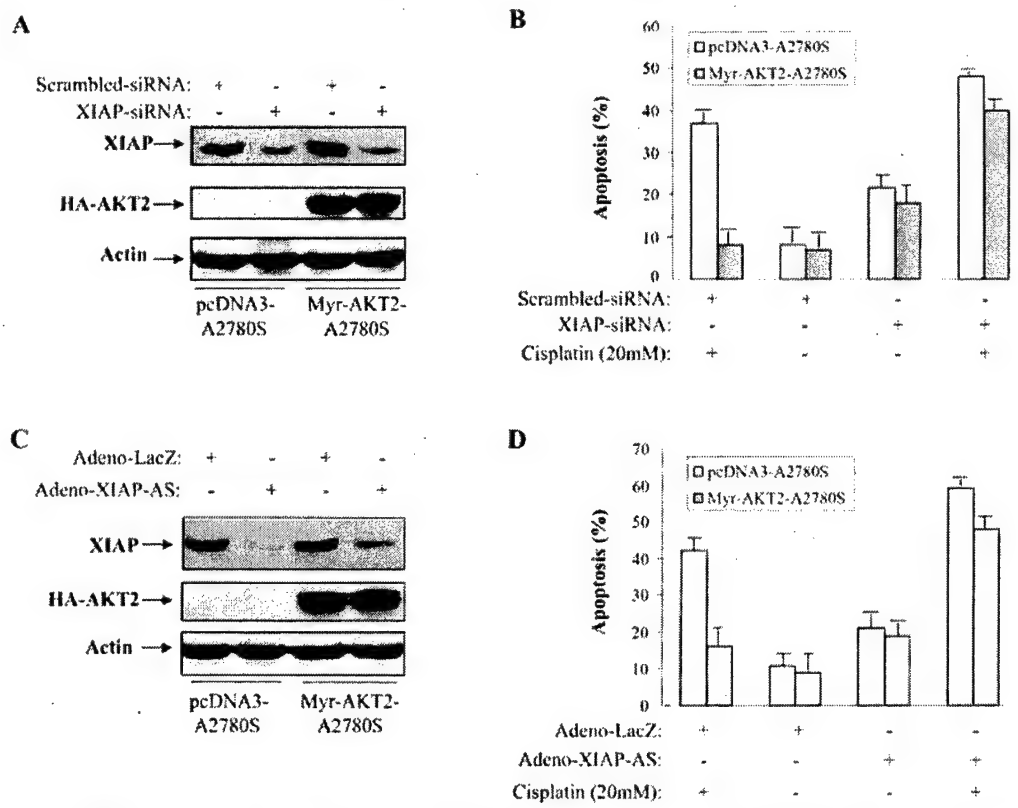

FIG. 6. **Inhibition of XIAP expression triggers apoptosis and abrogates Akt protection of cells from cisplatin-induced apoptosis.** Stable pcDNA3- and HA-Myr-AKT2-transfected A2780S cells were treated with siRNA (*A*) and adenovirus of antisense (*C*) of XIAP and subjected to Western blot analysis with anti-XIAP (*top*), -HA (*medium*), and -actin (*bottom*) antibodies. The apoptosis was examined with TUNEL assay (*B* and *D*). Each experiment was repeated three times.

ther demonstrate that Akt phosphorylates serine 87 of XIAP and that the inhibition of ubiquitination/degradation of XIAP depends on phosphorylation at this site. In addition, we have shown that Akt regulation of XIAP is not at transcriptional level (Figs. 1 and 2). However, we cannot exclude the possibility of Akt regulation of XIAP at translational level as Akt has been shown to activate mTOR/p70^{S6K} pathway (7). Nevertheless, these data indicate that the phosphorylation by Akt may be one of key mechanisms regulating XIAP levels and function in cells.

Accumulated evidence shows that Akt pathway exerts an anti-apoptotic action by regulation of molecules at both pre- and post-mitochondrial levels. The proapoptotic protein Bad has been shown to be a major target of Akt at the pre-mitochondrial level (13). However, the target(s) of Akt at the post-mitochondrial level have not been well documented, although a study demonstrated Akt phosphorylation and inhibition of caspase-9 (14). We show in this report that Akt interacts with and phosphorylates XIAP resulting in inhibition of caspase-3 activity and apoptosis in response to cisplatin treatment. Unlike caspase-9, in which Akt phosphorylation consensus site only exists in human (14, 33), the phosphorylation site of XIAP (^{82}**RHRKVS**^{87}) is well conserved among human, mouse, and rat (Fig. 4*A*). Thus, we conclude that XIAP could be a major target of Akt antiapoptotic function at post-mitochondrial level.

We have previously demonstrated that overexpression and/or activation of Akt contributes to cisplatin resistance in human ovarian cancer (24). XIAP content has been shown to be a determinant of cisplatin resistance in human ovarian cancer (21, 22). In this study, we have observed that cisplatin decreases XIAP protein content in A2780S cells without a significant change in XIAP mRNA level (Figs. 1 and 2) and suggest that *XIAP* gene transcription is not involved in XIAP down-regulation by cisplatin. Furthermore, ectopic expression of zinc RING finger domain deleted mutant XIAP, in which ubiquitin ligase activity was disrupted, rendered cells more resistant to cisplatin as compared with expression of wild type XIAP (34). These findings indicate that XIAP degradation is an important mechanism to regulate the steady-state XIAP and determine the sensitivity to cisplatin in human ovarian cancer cells. We demonstrate in this report that ectopic expression of constitutively active Akt protects XIAP from ubiquitination and degradation induced by cisplatin. Furthermore, phosphomimic XIAP-S87D is more resistant to ubiquitination/degradation induced by cisplatin than wild type XIAP. Conversely, the non-phosphorylatable XIAP-S87A shows increased ubiquitination relative to wild type XIAP (Fig. 5). In addition, suppression of XIAP by siRNA or antisense of XIAP abrogated Akt-induced cisplatin resistance and cell survival (Fig. 6). These data indicate that Akt stabilization of XIAP could be a major mechanism accounting for Akt-induced cisplatin resistance in human ovarian cancer cells.

In summary, the data presented here demonstrate for the first time that XIAP is regulated by phosphorylation. Akt phosphorylates XIAP at residue serine 87 *in vitro* and *in vivo* and interacts with XIAP at physiological protein concentration. The phosphorylation of XIAP at serine 87 by Akt results in inhibition of its autoubiquitination and ubiquitination and thus confers resistance to cisplatin-induced XIAP degradation, caspase-3 activation, and apoptosis. These results suggest that XIAP, in addition to Bad, is a major physiological substrate of Akt in the regulation of apoptosis, especially at the post-mitochondrial level.

Acknowledgment—We are grateful to the DNA Sequence Facility at the H. Lee Moffitt Cancer Center for sequencing XIAP mutant expression constructs.

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Oncogene (2004) 23, 706–715
 0950-9232/04 $25.00
www.nature.com/onc

Appendix 3

Phosphatidylinositol-3-OH kinase/AKT and survivin pathways as critical targets for geranylgeranyltransferase I inhibitor-induced apoptosis

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Geranylgeranyltransferase I inhibitors (GGTIs) represent a new class of anticancer drugs. However, the mechanism by which GGTIs inhibit tumor cell growth is still unclear. Here, we demonstrate that GGTI-298 and GGTI-2166 induce apoptosis in both cisplatin-sensitive and -resistant human ovarian epithelial cancer cells by inhibition of PI3K/AKT and survivin pathways. Following GGTI-298 or GGTI-2166 treatment, kinase levels of PI3K and AKT were decreased and survivin expression was significantly reduced. Ectopic expression of constitutively active AKT2 and/or survivin significantly rescue human cancer cells from GGTI-298-induced apoptosis. Previous studies have shown that Akt mediates growth factor-induced survivin, whereas p53 inhibits survivin expression. However, constitutively active AKT2 failed to rescue the GGTIs downregulation of survivin. Further, GGTIs suppress survivin expression and induce programmed cell death in both wild-type p53 and p53-deficient ovarian cancer cell lines. These data indicate that GGTI-298 and GGTI-2166 induce apoptosis by targeting PI3K/AKT and survivin parallel pathways independent of p53. Owing to the fact that upregulation of Akt and survivin as well as inactivation of p53 are frequently associated with chemoresistance, GGTIs could be valuable agents to overcome antitumor drug resistance.
Oncogene (2004) **23,** 706–715. doi:10.1038/sj.onc.1207171

Keywords: Akt; PI3K; survivin; GGTI; apoptosis; ovarian cancer

Introduction

Geranylgeranyltransferase I and farnesyltransferase inhibitors (GGTIs and FTIs) represent a new class of anticancer drugs (Sebti and Hamilton, 2000). These compounds were originally designed to block lipid post-translational modification of oncogenic Ras, which is essential for its function (Reiss *et al.*, 1990; Kohl *et al.*, 1993). Prenylation of small G proteins such as Ras, Rho, and Rac is critical to their cellular localization and function. Two types of prenyl transferases, farnesyltransferase and geranylgeranyltransferase (GGTase), have been shown to catalyse protein prenylation. FTase catalyses the transfer of farnesyl from farnesylpyrophosphate to a cysteine at the carboxyl terminus of proteins ending in CAAX, where C is cysteine and A is an aliphatic amino acid, and X is methionine, serine, cysteine, or glutamine. GGTase I, on the other hand, transfers geranylgeranyl from geranylgeranylpyrophosphate to CAAX terminal sequences, where X is leucine or isoleucine. We have developed CAAX peptidomimetics such as GGTI-298 and FTI-277 as highly selective inhibitors of GGTase I and FTase, respectively (Sebti and Hamilton, 1997). FTI-277 blocks potently oncogenic H-Ras processing and signaling. However, inhibition of the processing of K-Ras, the most prevalent form of mutated Ras in human tumors, becomes geranylgeranylated by GGTase I when FTase is inhibited. Therefore, both FTI-277 and GGTI-298 are required for inhibition of K-Ras processing in human tumor (Sebti and Hamilton, 1997). Several reports suggested that RhoB is a critical target for antitumor activity of FTIs (Du *et al.*, 1999; Prendergast, 2001). However, this remains controversial and other studies have provided evidences against inhibition of Rho-B farnesylation as a mechanism by which FTIs inhibit tumor cell survival and growth (Chen *et al.*, 2000).

Inhibitor of apoptosis proteins (IAPs) represent a conserved gene family that protects against programmed cell death induced by a variety of apoptotic stimuli (Deveraux and Reed, 1999). IAPs contain at least one BIR (baculovirus IAP repeat) domain that binds to caspases 3, 7, and 9 to inhibit their activities. Survivin is the smallest known IAP family protein and contains a single BIR domain with which it binds caspases and prevents caspase-induced apoptosis (Altieri, 2003). In addition, survivin also plays an important role in cell cycle control (Reed, 2001). Altered expression of

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Received 9 May 2003; revised 2 September 2003; accepted 3 September 2003

survivin appears to be a common event associated with the pathogenesis of human cancer; survivin is overexpressed in many transformed cell lines and in common cancers, such as those of the ovary, lung, colon, liver, prostate, and breast (Reed, 2001; Altieri, 2003). Reduced survivin expression causes apoptosis and sensitization to anticancer drugs, suggesting that survivin expression is important for cell survival or chemoresistance of certain carcinomas (Tran *et al*., 2002; Altieri, 2003).

Phosphatidylinositol-3-OH kinase/Akt is another major cell survival pathway that has been recently extensively studied (Brazil *et al*., 2002). PI3K is a heterodimer composed of a p85-regulatory and a p110-catalytic subunit and converts the plasma membrane lipid phosphatidylinositol-4-phosphate [PI(4)P1] and phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2] to phosphatidylinositol-3,4,-bisphosphate [PI(3,4)P2] and phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3]. Pleckstrin-homology (PH) domain-containing proteins, including Akt, accumulate at sites of PI3K activation by directly binding to PI(3,4)P2 and PI(3,4,5)P3. Akt (also known as PKB) represents a subfamily of the serine/threonine kinases. Three members of this family, including AKT1, AKT2, and AKT3, have been identified so far. Akt is activated by a variety of stimuli, including growth factors, protein phosphatase inhibitors, and stress in a PI3K-dependent manner (Franke *et al*., 1995; Datta *et al*., 1999). Several downstream targets of Akt, each of which contains the Akt phosphorylation consensus sequence R–X–R–X–X–S/T–F/L, have been identified (Datta *et al*., 1999), pointing to the possible mechanisms by which Akt promotes cell survival and blocks apoptosis. Akt phosphorylates the proapoptotic proteins BAD, caspase-9, and transcription factor FKHRL1, resulting in reduced binding of BAD to Bcl-X_L and inhibition of caspase-9 protease activity and Fas ligand transcription (Datta *et al*., 1999). Moreover, alterations of Akt, especially AKT2, have been frequently detected in human malignancy. Overexpression/activation of PI3K and/or Akt renders cancer cells resistant to conventional chemotherapeutic drugs (Cheng *et al*., 2002; Clark *et al*., 2002). It has also been shown that inactivation of PTEN and p53 results in constitutive activation of Akt pathway. PTEN mutations lead to loss of its lipid phosphatase activity, and thus, it is unable to convert PI(3,4,5)P3 to PI(4,5)P2 (Datta *et al*., 1999). p53 transcription factor has recently been found to bind to the promoters of PTEN and p110α to induce PTEN and inhibit p110α transcription. Therefore, mutations of p53 result in downregulation of PTEN and upregulation of p110α leading to activation of Akt (Stambolic *et al*., 2001; Singh *et al*., 2002).

In the present study, we demonstrate that GGTI-298 and GGTI2166 target PI3K/AKT2 and survivin pathways leading to programmed cell death in cisplatin-sensitive and -resistant human ovarian cancer cells via a p53-independent mechanism. Moreover, AKT1 activation was also inhibited by GGTI-298 and GGTI-2166. As AKT2, but not AKT1, is frequently altered in human cancer (Cheng *et al*., 1992; 1996; Yuan *et al*., 2000; Arboleda *et al*., 2003), we primarily focused our study on AKT2.

Results and discussion

GGTIs inhibit AKT2 and induce apoptosis in cisplatin-sensitive and -resistant human ovarian cancer cells

We have previously demonstrated that GGTI-298 arrests NIH 3T3 cells and lung cancer cells at G1 phase by upregulation of $p21^{WAF/CIP1}$ and hypophosphorylation of RB (Adnane *et al*., 1998; Sun *et al*., 1999a, b). We have also documented that GGTIs enhance the ability of FTIs to induce apoptosis in drug-resistant myeloma (Bolick *et al*., 2003) as well as synergize with other anticancer drugs such as cisplatin, taxol, and gemcitabine to inhibit human lung cancer cell growth in nude mice (Sun *et al*., 1999a, b). These results implicate the role of geranylgeranylated proteins in cell survival control, yet the involved mechanisms for inhibition of tumor growth and induction of apoptosis still remain unclear. Our previous studies showed that constitutively active H-Ras significantly activates PI3K/AKT2 and that the farnesyltransferase inhibitor, FTI-277, suppresses the PI3K/AKT2 pathway leading to cell death in human cancer cell lines (Liu *et al*., 1998; Jiang *et al*., 2000). These studies prompted us to examine the possible involvement of the PI3K/Akt pathway in GGTI antitumor activity. A cisplatin-sensitive (A2780S) and a cisplatin-resistant (A2780CP) ovarian cancer cell lines were treated with GGTI-298 (15 μM) or GGTI-2166 (20 μM) in DMEM supplemented with 10% FBS for 0, 12, 24, 36, and 48 h. Apoptosis and Akt activation were analysed by the Tunel assay and Western blot. Following GGTI-298 or GGTI-2166 treatment, both cisplatin-sensitive A2780S and cisplatin-resistant A2780CP cells underwent programmed cell death. Apoptotic cells reached approximately 70–80% after 36 h of treatment without significant difference between these two cell lines (Figure 1a and data not shown), indicating that GGTI-298 and GGTI-2166 are able to overcome cisplatin resistance in human ovarian cancer cells.

Immunoblotting analysis of AKT1 and AKT2 immunoprecipitates with phospho-Akt-Ser473 antibody revealed that GGTI-298 and GGTI-2166 inhibit phosphorylation of AKT1 and AKT2 after 12 h of treatment in both cisplatin-sensitive and -resistant cell lines. However, total AKT1 and AKT2 protein levels remained unchanged (Figure 1b and data not shown). These results suggest that GGTI may either directly or indirectly target Akt signal transduction pathway to induce apoptosis.

GGTIs target a geranylgeranylated protein(s) upstream of PI3K/AKT2 pathway

To demonstrate that GGTI-298 and GGTI-2166 actually suppress AKT2 kinase, A2780S cells were treated

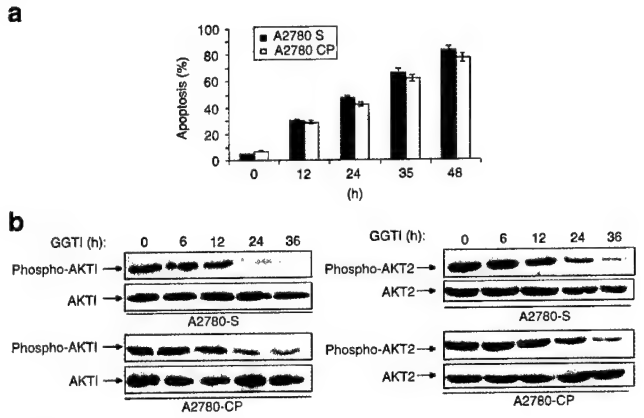

Figure 1 GGTI-298 inhibits Akt activation and induces apoptosis in cisplatin-sensitive and -resistant ovarian cancer cells. (**a**) Tunel assay. Cisplatin-sensitive A2780-S and cisplatin-resistant A2780-CP cells were cultured in DMEM supplemented with 10% FBS and treated with GGTI-298 (15 μM) for the indicated time. Apoptotic cells were detected with the Tunel assay and quantified. (**b**) Western blot analyses of the AKT1 (left) and AKT2 (right) immunoprecipitates prepared from A2780S and A2780CP cells following GGTI-298 treatment. The blots were detected with anti-phospho-Akt-Ser473 (panels 1 and 3), -AKT1 and -AKT2 (panels 2 and 4) antibodies. All the experiments were repeated three times

with or without EGF (50 ng/ml) for 15 min following treatment with GGTI-298 or GGTI-2166 for 12 h. *In vitro* kinase assays were then performed on AKT2 immunoprecipitates as described under Experimental procedures. As illustrated in Figure 2a, EGF-induced AKT2 kinase activity was abrogated by GGTI-298 treatment. As PI3K is an upstream activator of AKT2, we next examined whether GGTI-298 inhibits PI3K activity. Following GGTI-298 treatment and EGF stimulation as described above for AKT2 kinase assay, A2780S and A2780CP cells were immunoprecipitated with anti-pan-p85 antibody. PI3K activity was examined by *in vitro* kinase analysis of the immunoprecipitates using PI(4,5)P2 as a substrate. GGTI-298 attenuated EGF-stimulated PI3K activation (Figure 2c). However, GGTI-298 does not directly inhibit PI3K and AKT2 activities as determined by adding GGTI-298 to the kinase reaction *in vitro* (Figure 2b and d). In addition, GGTI-2166 exhibits the same

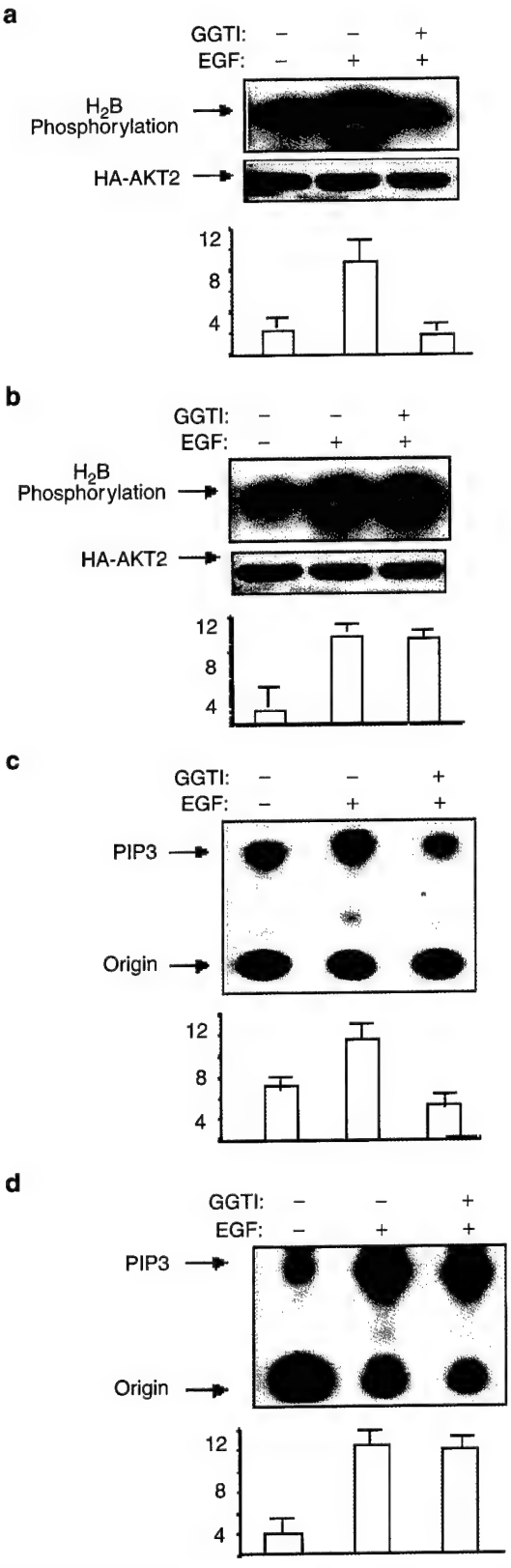

Figure 2 GGTI-298 inhibits PI3K and AKT2 activation. (**a**) *In vitro* kinase assay of the HA-AKT2 immunoprecipitates prepared from A2780S cells. After serum starvation overnight, the cells were treated with or without GGTI-298 for 12 h prior to EGF (50 ng/ml) stimulation for 15 min. Immunoprecipitation was performed with anti-AKT2 antibody and subjected to *in vitro* kinase assay using histone H2B as substrate. (**b**) GGTI-298 does not directly inhibit EGF-induced AKT2 activation. After serum starvation and stimulation with EGF, GGTI-298 (15 μM) was directly added into AKT2 kinase reaction. Following incubation for 30 min, the reactions were separated on SDS–PAGE gel and exposed to the film. (**c**) *In vitro* PI3K assay of the anti-p85 immunoprecipitates prepared from A2780CP cells. Following serum starvation overnight, the cells were treated with or without GGTI-298 for 12 h prior to EGF stimulation for 15 min. (**d**) GGTI-298 does not directly inhibit EGF-induced PI3K activation. *In vitro* PI3K assay of the PI3K immunoprecipitates derived from A2780CP cells. After serum starvation and stimulation, GGTI-298 (15 μM) was directly added to the kinase reaction. Quantification of AKT2 and PI3K activity from three repeated experiments is shown in bottom panels (**a–d**)

effects on PI3K/Akt activation as GGTI-298 (data not shown). These data imply that GGTI-298 and GGTI-2166 are not direct inhibitors of PI3K and AKT2 but rather target a geranylgeranylated protein(s) upstream of PI3K/AKT2 pathway.

Constitutively active AKT2 partially rescues A2780S cells from GGTI-induced apoptosis

We reasoned that if GGTI-298 and GGTI-2166 inhibit a geranylgeranylated protein upstream of PI3K/AKT2, then constitutively active AKT2 should overcome GGTIs-induced apoptosis. A constitutively active AKT2 expression construct (HA-Myr-*AKT2*) or pcDNA3 vector alone was stably transfected into A2780S cells. Western blot analysis with anti-HA antibody revealed expression of HA-Myr-AKT2 in the transfectants (Figure 3a). After treatment with GGTI-298 (15 μM) or GGTI-2166 (20 μM) for different times in the presence of 10% FBS, apoptotic cells were observed in pcDNA3- and Myr-AKT2-transfected A2780S cells. The percentages of apoptotic cells in pcDNA3-transfected A2780S cells increased from 8% at time 0 to 80% after 48 h of treatment with GGTI-298 (Figure 3b). These percentages are very similar to those reported in Figure 1a for nontransfected parental A2780S cells. In contrast, GGTI-298 induced apoptosis by 40% at time 48 h of treatment in cells transfected with constitutively activated AKT2. Similar effects were observed in the cells treated with GGTI-2166 (data not shown). Therefore, constitutively active AKT2 only partially rescues A2780S cells from GGTI-induced apoptosis (Figure 3b), indicating that other cell survival signal molecule(s) must be targeted by GGTI-298 and GGTI-2166 besides PI3K/Akt pathway.

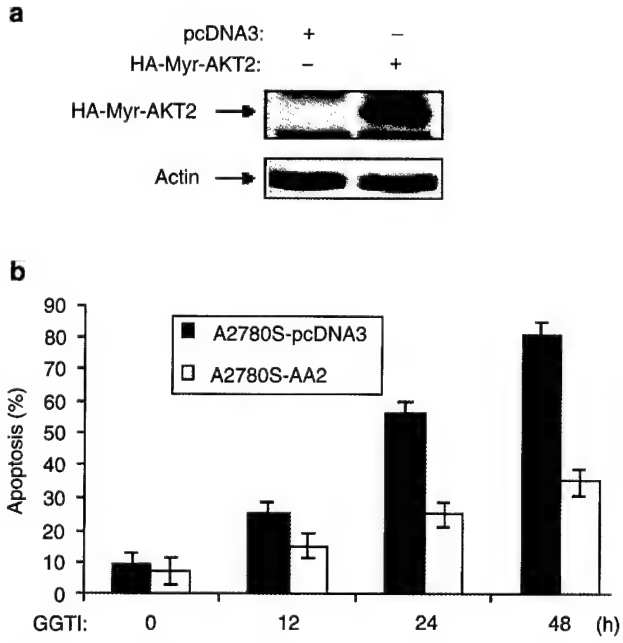

Figure 3 A constitutively activated form of AKT2 partially rescues A2780S cells from GGTI-298-induced apoptosis. (**a**) A2780S cells were stably transfected with constitutively active AKT2 (Myr-AKT2, AA2). Western blot analysis with anti-HA antibody revealed expression of transfected HA-Myr-AKT2 in a clonal cell line (upper panel). Bottom panel shows equal loading. (**b**) Tunel assay. After treatment of A2780S-pcDNA3 and A2780S-AA2 cells with GGTI-298 for the indicated times, apoptotic cells were detected and quantified from three independent experiments

GGTIs downregulate the IAP family protein survivin

Numerous studies have shown that IAP family proteins play a critical role in cell survival (Deveraux and Reed, 1999; Reed, 2001; Tran *et al*., 2002; Altieri, 2003). Among the members of IAP family, only survivin is frequently overexpressed in human cancer including ovarian carcinoma and ectopic expression of survivin renders ovarian cancer cells resistant to taxol (Zaffaroni *et al*., 2002). Thus, we next examined whether GGTI-298 or GGTI2166 targets survivin to induce apoptosis in ovarian cancer cells. A2780S cells, in which survivin is highly expressed, were treated with GGTI-298 or GGTI-2166 for different times. Western and Northern blot analyses revealed that both protein and mRNA levels of survivin were significantly reduced following GGTI-298 or GGTI-2166 treatment (Figure 4a and data not shown). To further examine the importance of survivin in GGTIs proapoptotic activity, A2780S cells were stably transfected with Myc-tagged survivin. Again, the cells transfected with pcDNA3 vector alone were used as control. Expression of transfected Myc-survivin was confirmed by immunoblotting analysis with anti-Myc antibody (Figure 4c). Following administration of GGTI-298 or GGTI-2166 at various lengths of time, apoptotic cells were detected by the Tunel assay and quantified. Both GGTI-298 and GGTI-2166 increased apoptosis from 8 and 10% at time 0 to 70 and 80% at time 24 h in A2780S-pcDNA3 cells, respectively. In survivin-expressing cells, both inhibitors induced apoptosis to only 30% after 24 h of treatment (Figure 4d and data not shown). Thus, ectopic expression of survivin rescues the cells from GGTI-induced apoptosis but only partially, implying that survivin is another target of GGTIs in addition to PI3K/AKT2. Further, A2780S cells were stably contransfected with constitutively active AKT2 and survivin (Figure 4c) and treated with either GGTI-298 (15 μM) or GGTI-2166 (20 μM). The Tunel assay analysis revealed that cells expressing both myr-AKT2 and survivin became dramatically resistant to GGTIs treatment (Figure 4d), indicating that AKT2 and survivin are critical targets of GGTIs at least in A2780S ovarian cancer cells.

GGTIs inhibit survivin via a p53-independent pathway

Previous investigations have demonstrated that p53 represses survivin expression through inhibiting its transcription (Hoffman *et al*., 2002; Mirza *et al*., 2002). To determine whether GGTIs' suppression of survivin expression depends on p53, we evaluated the effects of GGTIs on survivin expression in A2780CP cells that carry p53 mutation (Sasaki *et al*., 2000). A2780CP cells were cultured in DMEM supplemented

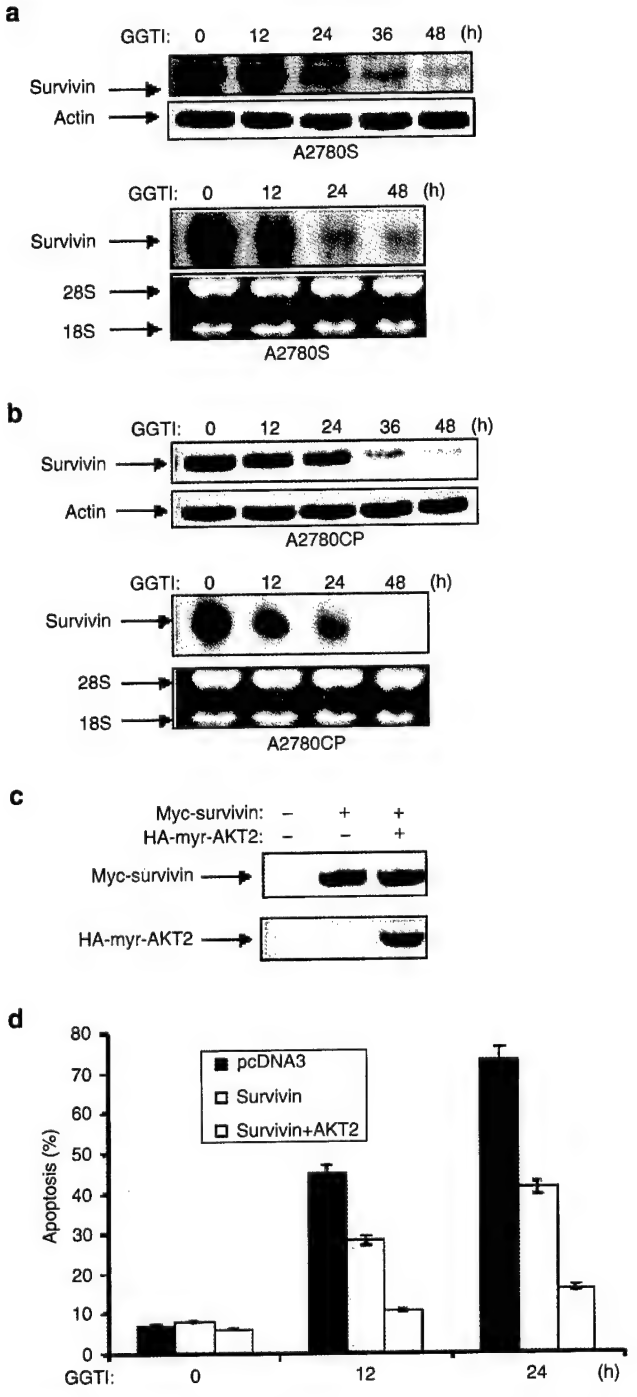

Figure 4 GGTI-298 inhibits expression of survivin independent of p53 pathway. (**a** and **b**) Western (upper panels) and Northern (lower panels) analyses of expression of survivin in A2780S (wild-type p53) and A2780CP (mutant p53) cells treated with GGTI-298 at indicated time. Northern blot analysis with [^{32}P]dCTP-labeled survivin cDNA probe (upper). Equal loading of total RNA was shown in bottom panel. (**c**) Immunoblotting analysis of expression of transfected Myc-survivin and HA-myr-AKT2 in A2780S cells with anti-Myc (upper) and anti-HA (bottom) antibodies. (**d**) Tunel assay. Following treatment of A2780S-pcDNA3, A2780S-survivin and A2780S-survivin/myr-AKT2 cells with GGTI-298 at indicated time, apoptotic cells were detected with the Tunel assay and quantified

with 10% FBS and treated with GGTI-298 or GGTI-2166 for different times. The expression of survivin was evaluated by Western and Northern blot analyses. Both protein and mRNA levels of survivin were inhibited by GGTI-298 and GGTI2166 treatment in A2780CP cells (Figure 4b and data not shown). Quantification analysis showed that GGTI-inhibited survivin expression was similar in A2780CP cells that contain mutant p53 and A2780S cells that express wild-type p53 (Figure 4a and b). To further define the effects of p53 on GGTIs' suppression of survivin expression, A2780CP cells were stably transfected with HA-tagged wild-type p53 and pcDNA3 vector alone, as a control. Figure 5a shows that transfected p53 expresses and is functional reflected by elevated level of p21^{WAF1} and restoration of A2780CP

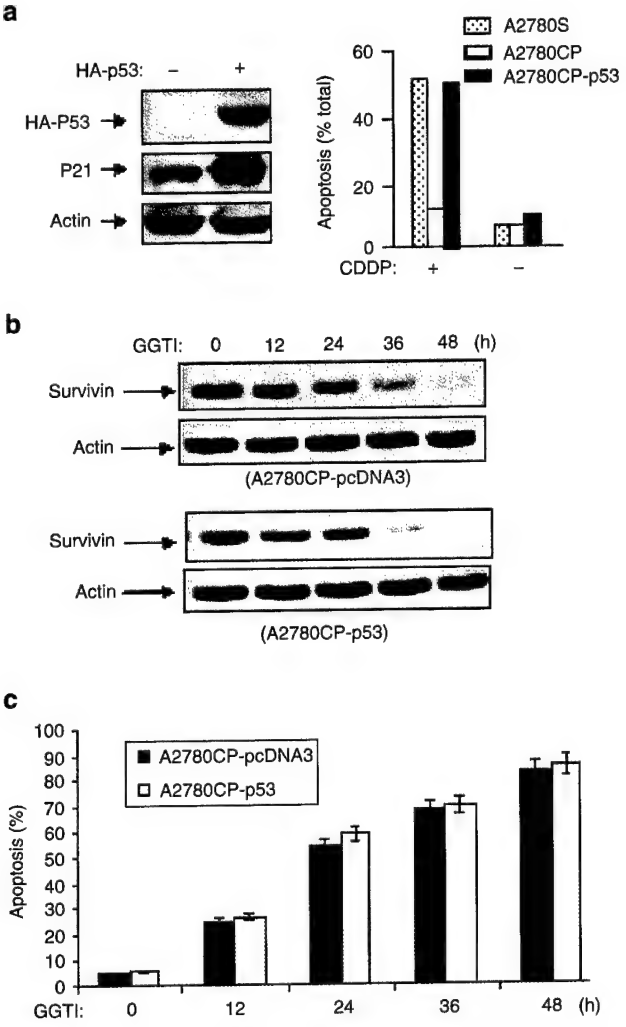

Figure 5 Ectopic expression of p53 did not affect GGTI action. (**a**) Immunoblotting analysis of expression of transfected wild-type HA-p53 in A2780CP cells with anti-HA (top) and anti-p21 (middle) antibodies. The bottom panel showed equal loading. (**b**) Immunoblotting analysis of survivin expression in pcDNA3- (upper panels) and HA-p53-transfected (bottom panels) A2780CP cells. (**c**) Reintroduction of wild-type p53 into A2780CP cells did not sensitize the cells to GGTI-298-induced apoptosis. Following GGTI-298 treatment at indicated time, apoptotic cells were detected and quantified from three independent experiments

cells sensitive to cisplatin treatment. Immunoblotting analysis showed that reintroduction of wild-type p53 into A2780CP cells did not have significant effects on the ability of GGTIs to inhibit survivin expression as compared to pcDNA3-transfected A2780CP cells (Figure 5b). These results indicate that GGTIs suppression of survivin is independent of p53 pathway.

Previous studies have shown that re-expression of wild-type p53 sensitizes A2780CP cells to cisplatin-induced apoptosis (Song *et al*., 1997; Sasaki *et al*., 2000). Therefore, we next examined whether ectopic expression of wild-type p53 sensitizes A2780CP cells to GGTI-stimulated cell death. The Tunel assay revealed that the levels of GGTI-induced apoptosis were the same in A2780CP-p53, A2780CP-pcDNA3 as well as A2780S cells (Figures 5c and 1a). We have previously shown GGTI-298-mediated transcriptional upregulation of $p21^{WAF1/CIP1}$ is also independent of p53 (Adnane *et al*., 1998). This further supports the notion that the mechanism of GGTIs antitumor activity does not involve the p53 pathway. Since mutant p53 is a major contributor to anticancer drug resistance and since GGTIs can overcome this resistance at least in the case of cisplatin, combination of GGTIs with these agents has a potential for cancer treatment.

GGTIs attenuated AKT2-induced survivin expression and promoter activity

Recent studies have shown that PI3K/Akt pathway mediates IGF1- and VEGF-upregulation of survivin protein in multiple myeloma and endothelial cells (Papapetropoulos *et al*., 2000; Mitsiades *et al*., 2002). However, the underlying molecular mechanism has not been well documented. As GGTI inhibits PI3K/AKT activation as well as survivin expression at the transcription level, we reasoned that activation of AKT2 could induce survivin transcription. To this end, Northern blot analysis of A2780S cells transfected with constitutively active AKT2 revealed that expression of survivin was induced by AKT2 in a dose-dependent manner (Figure 6a). Further, a luciferase activity assay was carried out with HEK293 cells transfected with pGL3-survivin-Luc reporter, constitutively active AKT2 and β-galactosidase. Triple experiments showed that ectopic expression of constitutively active AKT2 stimulated survivin promoter activity (Figure 6b). These data indicate that AKT2 upregulates survivin by inducing its promoter activity. It has been demonstrated that survivin promoter contains a NFκB-binding site and is induced by NFκB pathway (Deveraux and Reed, 1999; Mitsiades

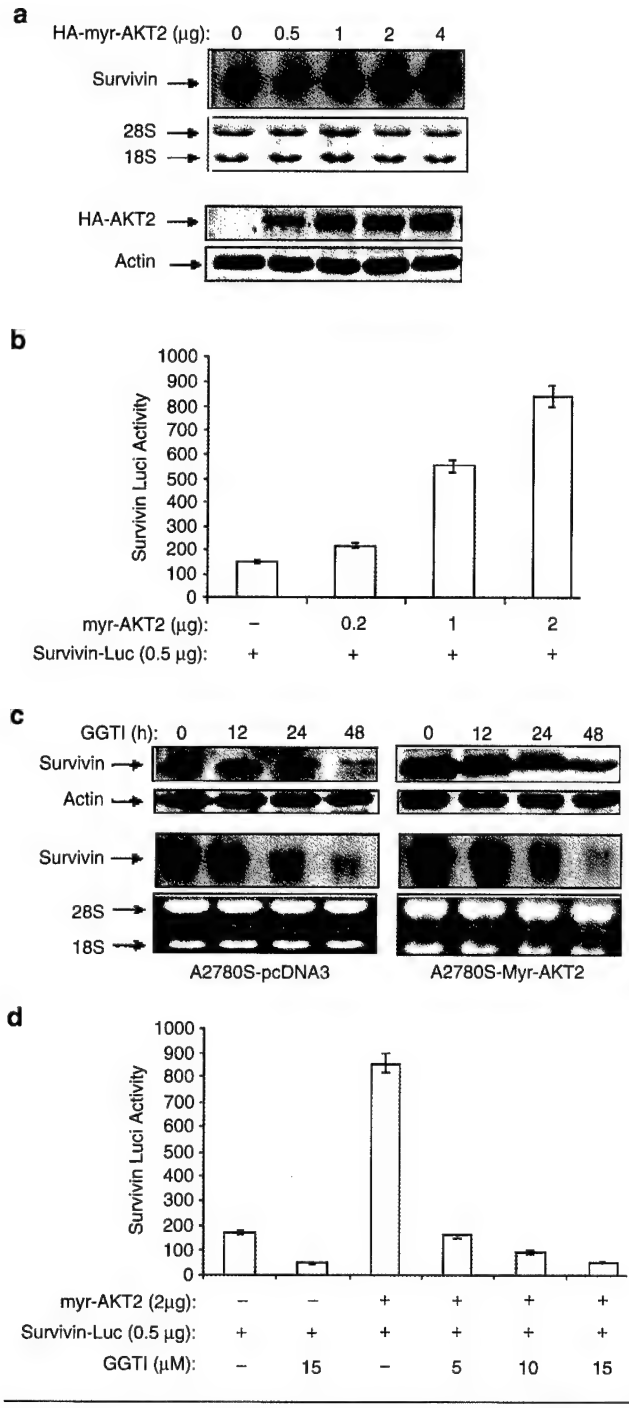

Figure 6 Constitutively active AKT2 induces survivin transcription and promoter activity; AKT action failed to rescue GGTI-downregulated survivin. (**a**) Northern blot analysis of A2780S cells transfected with indicated amount of constitutively active AKT2. The blot was probed with [^{32}P]dCTP-labeled survivin cDNA (upper panel). Equal loading was shown in panel 2. Expression of transfected constitutively active AKT2 was detected with anti-HA antibody (panel 3). The same blot was reprobed with antiactin antibody (bottom panel). (**b**) Luciferase reporter assay. HEK293 cells were transfected with indicated plasmids. After 36 h of the transfection, luciferase and β-galactosidase assays were performed and the reporter activity was normalized by dividing luciferase activity with β-galactosidase. (**c**) Western (panels 1 and 2) and Northern (panels 3 and 4) blot analyses of pcDNA3- and constitutively active AKT2-trnasfected A2780S cells following treatment with GGTI-298 at indicated time. Western blots were detected with anti-survivin (upper) and anti-actin antibodies (panel 2). Northern blots were probed with [^{32}P]dCTP-labeled survivin (panel 3). Equal RNA loading was shown in bottom panels. (**d**) The luciferase reporter assay was performed as described in (**b**), except the cells were treated with indicated concentrations of GGTI-298 for 6 h prior to assay for luciferase and β-galactosidase activity. Each experiment was repeated three times

et al., 2002). We and others have shown that AKT1 and AKT2 activate the NFκB pathway through interaction and phosphorylation of IKKα and Cot/Tpl2 (Ozes *et al*., 1999; Madrid *et al*., 2000; Kane *et al*., 2002; Yuan *et al*., 2002). Therefore, AKT2-induced survivin transcription could be mediated by activation of this pathway.

Since AKT2 upregulates survivin and GGTIs repress survivin expression and AKT2 activity, one possible mechanism by which GGTIs could repress survivin is through inhibition of PI3K/AKT2. To test this hypothesis, constitutively active AKT2- and pcDNA3-stably transfected A2780S cells were treated with GGTI-298 or GGTI-2166. Following the treatment for 12, 24, and 48 h, expression of survivin was examined by Western and Northern blot analyses. As shown in Figure 6c, both basal protein and mRNA levels of survivin were higher in A2780S-Myr-AKT2 cells as compared to A2780S transfected with pcDNA3 vector alone. However, declining rate of the survivin induced by GGTIs was essentially the same between constitutively active AKT2- and pcDNA3-transfected A2780S cells. Moreover, the luciferase reporter assay showed that constitutively active AKT2-stimulated survivin promoter activity was also attenuated by GGTIs treatment. Even the basal levels of survivin promoter activity were significantly inhibited by GGTI-298 or GGTI-2166 (Figure 6d and data not shown). As GGTI-298 and GGTI-2166 are not direct AKT2 inhibitor (Figure 2c), we conclude that GGTIs repress survivin by targeting other molecule(s), which bypasses AKT2 but is capable of blocking AKT2-induced survivin transcription. Moreover, these data also indicate that GGTIs induce apoptosis in human ovarian cancer cells by inhibition of survivin and PI3K/AKT2 parallel pathways.

Effects of FTI and/or GGTI on apoptosis, AKT activity, and survivin expression

To examine whether GGTI antitumor activity is mediated by decrease in protein geranylgeranylation or a compensatory increase in protein farnesylation, we cotreated A2780S cells with FTI and GGTI. The cells treated with FTI and GGTI alone were used as controls. Triple experiments revealed that FTI induces apoptosis at much lesser extent than GGTI. The apoptosis induced by cotreatment with GGTI and FTI is higher than that of either GGTI or FTI alone; however, FTI did not exhibit dramatic effects on GGTI-induced apoptosis in A2780S cells (Figure 7a). Immunoblotting analysis showed that GGTase I substrate Rap1A and of FTase substrate HDJ2 were inhibited by GGTI-298 and FTI-277 in A2780S cells, respectively (Figure 7b). Moreover, GGTI-downregulated survivin was not affected by FTI treatment, even though phosphorylation level of Akt was inhibited by GGTI/FTI at higher degree as compared to GGTI or FTI alone (Figure 7c). These data suggest that FTI and GGTI have no significant synergic inhibitory effects on cell survival and survivin expression in A2780S cells and that GGTI treatment did not result in a compensatory increase in protein farnesylation.

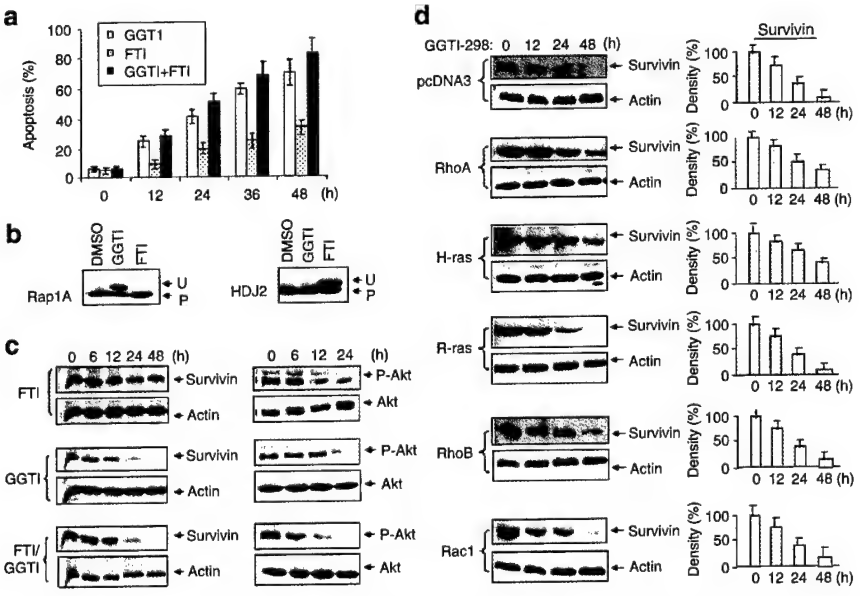

Figure 7 Effects of FTI and GGTI on cell survival, Akt activation and expression of survivin. (**a**) Tunel assay. A2780S cells were treated with GGTI-298 (15 μM) together with FTI-277 (20 μM) and GGTI or FTI alone. After treatment for indicated time, apoptotic cells were detected with Tunel assay. (**b**) A2780S cells were treated as panel (**a**) and subjected to Western blot analysis with anti-Rap1 and -HDJ2 antibodies. *U* and *P* designate unprocessed and processed forms of Rap1 and HDJ2. (**c**) Immunoblotting analysis of A2780S cells treated as described in panel (**a**) with indicated antibodies. (**d**) A2780S cell were transiently transfected with indicated plasmids using LipofactAmine Plus. Approximate 70% transfection efficiency was achieved using transfection of EGFP-C2 vector as an indicator. After 36 h of transfection, cells were treated with GGTI-298 (15 μM) and then immunoblotted with indicated antibodies (left panels). Right panels show the quantification of survivin protein levels from three independent experiments

As GGTI was originally designed to inhibit small G-proteins, we next examined whether ectopic expression of small G-protein(s) over-rides GGTI-dowregulated survivin. A2780S cells were individually transfected with expression constructs of v-H-Ras, R-Ras, Rac, RhoB, and RhoA. After 36 h of transfection, the cells were treated with GGTI-298 for different time, expression of survivin was analysed by Western blot. As shown in Figure 7d, survivin was significantly declined in GGTI-298-treated pcDNA3-transfected cells. However, none of small G-proteins examined has dramatic protection from GGTI downregulation of survivin even though H-ras and RhoA slightly inhibit GGTI-induced survivin declining rate. These data suggest that these small G-proteins do not seem to be the targets of GGTI to downregulate survivin in A2780S cells even though Akt has been shown to be activated by some of them (Datta *et al*., 1996; Liu *et al*., 1998).

In summary, the data presented here demonstrate for the first time that GGTI-298 and GGTI-2166 potently inhibit PI3K/AKT2 activation and survivin expression in both cisplatin-sensitive and -resistant human ovarian cancer cell lines. Furthermore, our data suggest a mechanism by which GGTIs repress survivin expression by showing that GGTI-298 inhibits mRNA and promoter activity of survivin independent of p53 status and AKT2 activation (Figure 8). Finally, we provide evidence that GGTI-induced apoptosis is independent of p53 pathway. Since upregulation of Akt and survivin as well as inactivation of p53 are frequently associated with chemoresistance, GGTIs could be valuable agents to overcome antitumor drug resistance. Further investigations are required to characterize the mechanism by which GGTIs downregulate survivin and inactivate PI3K, that is, identification of GGTI-298- and GGTI-2166-targeted geranylgeranylated proteins that positively regulate PI3K/Akt and survivin pathways independently (Figure 8).

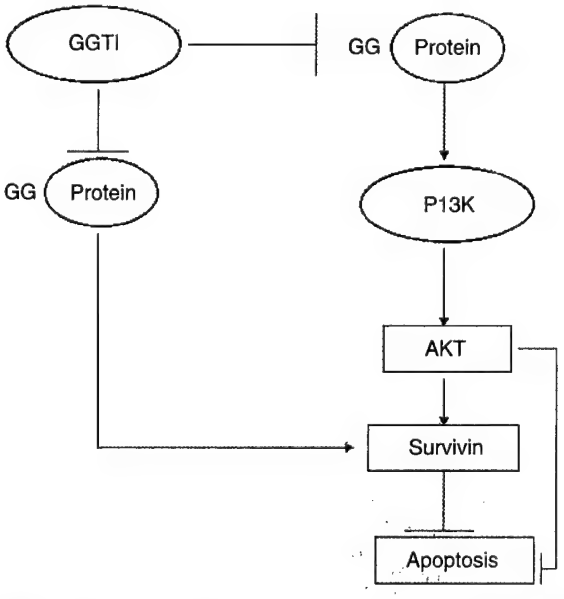

Figure 8 Schematic illustration of the mechanism of GGTI-298 induction of apoptosis in human cancer cells

Material and methods

Cell lines, transfection, and cell treatment

Human ovarian epithelial cancer cell lines A2780S and A2780CP and human embryonic kidney (HEK) 293 were cultured at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS. The cells were seeded in 60-mm Petri dishes at a density of 0.6×10^6 cells/dish and were transfected with 2 μg of DNA per dish using LipofecAMINE Plus. Stable clonal cell lines were established by G418 (500 μg/ml) selection.

Expression constructs

HA-AKT2 and HA-Myr-AKT2 were prepared as described previously (Jiang *et al*., 2000). HA-tagged p53 was prepared by releasing p53 from GST-p53 plasmid, kindly provided by Jiandong Chen at H Lee Moffitt Cancer Center, and cloning to HA-pcDNA3.1 vector. Survivin expression plasmid was created by PCR, subcloned to Myc-tagged pcDNA3.1 and confirmed by sequencing analysis. Based on published sequence (Li and Altieri, 1999), survivin promoter (−1469/+20) was amplified by PCR using normal human placenta genomic DNA as template. The PCR products were ligated into *Bam*HI–*Sma*I sites of pGL3 vector. The promoter sequence was confirmed by DNA sequencing.

Tunel assay

Cells were seeded into 60 mm dishes and grown in 10% FBS–DMEM for 36 h. Cells were then treated with 15 μM GGTI-298 for different times ranging from 0 to 48 h. Apoptosis was determined by terminal Tunel assay using an *in situ* cell death detection kit (Boehringer Mannheim, Indianapolis, IN, USA). The cells were trypsinized, and cytospin preparations were obtained. Cells were fixed with freshly prepared paraformaldehyde (4% in PBS, pH 7.4). Slides were rinsed with PBS, incubated in permeabilization solution, followed by Tunel reaction mixture for 60 min at 37°C in a humidified chamber. After a rinse, the slides were incubated with converter-alkaline phosphatase solution for 30 min at 37°C and then detected with alkaline phosphatase substrate solution (Vector Laboratories, Burlingame, CA, USA). After an additional rinse, the slides were mounted and analysed under a light microscope. These experiments were performed in triplicate.

Immunoprecipitation, in vitro kinase assay, Western and Northern blotting analyses

Following stimulation and treatment with GGTI, cells were lysed and immunoprecipitated with anti-AKT2 or Ant-HA antibody. The immunoprecipitates were sub-

jected to *in vitro* kinase assay using histone H2B as substrate. Protein expression was determined by probing Western blots with the appropriate antibodies. For the detection of endogenous phospho-AKT2, Western blot analysis of the AKT2 immunoprecipitates was performed and detected with anti-phospho-Akt-Ser473 antibody. Detection of antigen-bounded antibody was carried out with the ECL Western Blotting Analysis System (Amersham). Northern blot was performed as previously described (Cheng *et al.*, 1992).

PI3K assay

PI3K was immunoprecipitated from the cell lysates with anti-pan-p85 antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed once with cold PBS, twice with 0.5 M LiCl/0.1 M Tris (pH 7.4), and finally with 10 mM Tris/100 mM NaCl/1 mM EDTA. The presence of PI3K activity in immunoprecipitates was determined by incubating the beads in reaction buffer (10 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 50 μM ATP) containing 20 μCi [-^{32}P]ATP and 10 μg L-α-phosphatidylinositol 4,5-bis phosphate (Biomol) for 20 min at 25°C. The reactions were stopped by adding 100 μl of 1 M HCl. Phospholipids were extracted with 200 μl $CHCl_3$/MeOH and phosphorylated products were separated by thin-layer chromatography as previously described (Jiang *et al.*, 2000). The conversion of PI (4,5)P_2 to PI(3,4,5)P_3 was detected by autoradiography.

Luciferase reporter assay

Cells were seeded in six-well plate and transfected with survivin-Luc reporter, pSV2-β-gal and, constitutively active of AKT2. After 36 h of transfection and treatment with or without GGTI-298, luciferase and β-galactosidase assays were performed according to the manufacturer's procedures (Promega and Tropix), respectively. Each experiment was repeated three times.

Acknowledgements
We are grateful to Jian-dong Chen for GST-p53 plasmid and Benjamin K Tsang for ovarian cancer cell lines. We are also grateful to the Molecular Biology DNA Sequence Facility at H Lee Moffitt Cancer Center for sequencing Survivin constructs. This work was supported by National Cancer Institute Grants CA085709, CA67771, CA89242 and Department of Defense DAMD 17-01-1-0394 and DAMD17-02-1-0670.

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Curriculum Vitae

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Fox Chase Cancer Center, Philadelphia	(Staff Scientist)	1996-1997
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Research Grants and Awards (Total cost, 45% indirect):

1. Disruption of AKT survival pathway for cancer intervention — 2004.8 – 2009.8
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   Principal Investigator

2. AKT2 oncogene and human oncogenesis
   NIH R01 CA77935 — 2003.6-2008.6
   Principal Investigator — $1,205,315.00

3. AKT1 oncogene in carcinogenesis
   NIH R01 CA089242 — 2001.6-2006.6
   Principal Investigator — $1,141,875.00

4. AKT survival pathways and FTI-induced apoptosis
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1. Excellent in Teaching and Mentorship Award from University of South Florida — 2003
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Publications:

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